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PULSATION OF THE CONTRACTILE VACUOLE OF PARAMECIUM AS AFFECTED BY TEMPERATURE.

By WILLIAM H. COLE.

(*From the Biological Laboratory of Clark University, Worcester.*)

(Accepted for publication, February 25, 1925.)

In view of the recent investigations on the effect of temperature changes on biological processes (Crozier, 1924–25, *a*, *b;* Crozier and Federighi, 1924–25; Crozier and Stier, 1924–25, *a*, *b;* Glaser, 1924–25) it became of interest to reexamine the relation between temperature and the rate of pulsation of the contractile vacuole of *Paramecium*. Kanitz (1907) found in four different species of Infusoria that as the temperature was raised, the rate of pulsation of their contractile vacuoles increased, agreeing with the work of earlier authors, and Khainsky (1911) reported similar results with *Paramecium caudatum*. Such an increase in rate is to be expected, since the function of the contractile vacuole is chiefly to expel the waste products of catabolism, arising from oxidative processes which are accelerated by increased temperatures. If the rate of pulsation is determined solely by the rate of these oxidative processes, then the thermal increments, or temperature characteristics, for pulsation might have the values typical of other oxidative processes (Crozier, 1924–25, *b*). If other factors are also involved the increments might be different.

Observations have been made on *Paramecium caudatum* in order to determine the thermal increments for vacuole pulsation, which should assist in identifying the underlying and determining chemical reactions. The animals were secured from wild cultures, and were grown in hay infusions of constant concentration. Besides the *Paramecia*, which were always predominant, the cultures contained also smaller ciliates, such as *Colpidium* and *Euplotes*, and a few small rotifers. When animals from such cultures were studied it was found that the pulsation rate varied greatly in different individuals at the same temperature, although the rates obtained from

one individual were nearly constant. In some cases the posterior vacuole showed a higher rate than the anterior, while in other cases, the reverse was true. It was necessary, therefore, to study one vacuole of a single individual at different temperatures. The incessant activity of *Paramecium* rendered the observation of one animal for more than a few seconds or minutes unsatisfactory, and in most cases impossible. Various methods for quieting the animals without exposing them to unfavorable stimuli were tried without much success. Finally, the chloretone method[1] was employed, with the hope that the thermal increment for pulsation would not be affected, although the actual rate of pulsation might be influenced.

The slide bearing the observation chamber was placed in a micro slide incubator, insulated at the top and bottom, and containing a tested thermometer. By opening the windows of the room in which the observations were made, it was possible to secure the lower temperatures. After closing the windows, the room temperature returned to normal so slowly (in about 2 hours) that readings could be taken at intervening points. The higher temperatures were obtained by allowing the electric current to flow through the heating coil of the incubator as long as necessary. Between 9° and 31°C. the rates of pulsation at particular temperatures are constant, whether they be obtained from decreasing or increasing temperatures. The average pH of the various cultures at the beginning of the experiments was 7.6. At the end of 24 hours it fell to 7.2. It is not believed, therefore, that the hydrogen ion concentration increased to more than pH 7.4 during the first 12 hours of confinement.

After the animals had been confined for about 2 hours, single individuals were selected, normal in every visible respect, and the rate of pulsation of the anterior contractile vacuole was determined at temperatures from 9–31°C. The time required for one complete pulsation (from the beginning of one contraction to the beginning of the next one) was measured by a stop-watch. Five readings were taken at each temperature for each animal when little variation appeared, while ten readings were taken when the variation was

[1] The animals were confined in sealed glass chambers filled with culture fluid and chloretone solution. The resulting concentration of chloretone was about 0.056 per cent. For details of the procedure, see Cole and Richmond (1924–25).

sufficient to make this desirable. The average rates per minute for each animal were then calculated separately.

TABLE I.

Average Rates of Pulsation of the Anterior Contractile Vacuole of One Paramecium at Different Temperatures.

Temperature.	Average time for one pulsation.	Rate: pulsations per min.	Rates reported by Khainsky.
°C.	*sec.*		
9	94.7	0.63	
10	77.5	0.77	
11	74.1	0.81	
12	53.0	1.13	
13	48.4	1.24	
14	40.0	1.50	
15	37.7	1.59	
16	30.7	1.95	2.86
17	24.6	2.43	3.16
17.25	25.8	2.32	
18			3.33
18.5	22.5	2.66	
19			3.75
19.5	20.5	2.99	
20			4.29
21	17.2	3.49	4.81
22			5.50
22.25	15.0	4.00	
22.75	15.3	3.92	
23			6.00
23.25	14.4	4.16	
23.75	14.1	4.26	
24			6.00
25	13.2	4.54	6.00
25.5	12.5	4.80	
26			6.66
27			6.66
28			7.50
28.25	11.1	5.41	
29			7.50
30	10.6	5.66	8.55
31	9.5	6.32	8.55

Table I shows the data from one individual over a temperature range of 22 degrees (9–31°C.). The observations were begun 2½

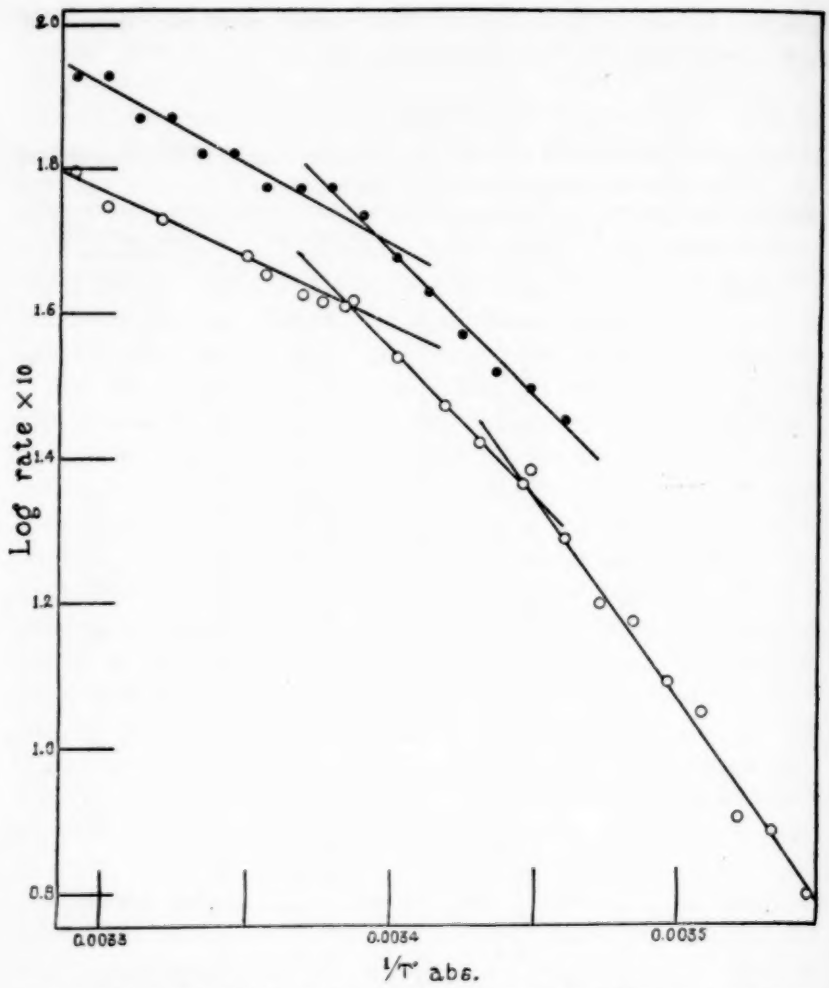

FIG. 1. Average pulsation rates of the anterior contractile vacuole of *Paramecium* at different temperatures. The rates have been multiplied by 10 and their logarithms plotted against the reciprocals of the absolute temperatures. The upper curve (black circles) is drawn from Khainsky's data; the lower one (white circles) from results described in the present paper. Below 16° the thermal increment (μ) is 25,600; between 16° and 22°, it is 18,600 and 18,900 (Khainsky's data); and from 22–31° it is 8,600 and 10,200 (Khainsky's data). In each case the value was determined from the slope of the line which best fitted the points plotted.

hours after confinement, and were continued for the 10 succeeding hours. During this time there were no evidences of morphological or physiological abnormalities in the animal, which fully recovered when placed in fresh culture fluid. In the last column of the table are given the rates reported by Khainsky, which, if divided by a constant (1.35), will yield values very near to those in the third column. Both sets of data are plotted in Fig. 1, where the rate of pulsation is shown to be a logarithmic function of temperature, according to the Arrhenius equation,[2]

$$K_2 = K_1 e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

Although the plot of Khainsky's data does not coincide with mine, the slope of the lines is, nevertheless, nearly the same, μ having the values 10,200 and 8,600 respectively, above 22°, and 18,600 and 18,900 between 17° and 22°. Below 16° there are no figures from Khainsky, but my data show μ to be about 25,600. All of these values were calculated from the slopes of the lines. Other series of observations on single animals resulted in different rates of pulsation at the same temperatures. When they were plotted, however, the slopes of the lines were the same, showing that the thermal increments were the same in all cases. If vacuole pulsation is dependent upon chemical reactions which are catalyzed, then the suggestion of Loeb and Chamberlain (1915), that individual variations in their rates may be caused by varying amounts of the effective catalyst present in different individuals, may be applied in this case.

Since the pulsation rates between 16° and 31° show essentially the same thermal increments for normal (Khainsky) and for chloretonized animals, it may be assumed that over this range of temperatures chloretone does not affect the temperature characteristic of pulsation frequency. Below 16° there are no reliable data for *Paramecium* in its normal environment. Although none of the increments agree exactly with those found by Crozier to be characteristic of oxidative processes, namely 7,900, 11,500, and 16,500 (Crozier, 1924–25, *b;* Crozier and Stier, 1924–25), this does not mean necessa-

[2] The theoretical considerations involved in the use of this equation are discussed in the papers of Crozier and Glaser already referred to.

rily that oxidations are not primarily responsible for the pulsation in *Paramecium*. Other unknown reactions may modify the oxidative ones, or may independently be the slow controlling processes. It is impossible to conclude that the rate of respiration in *Paramecium* is solely responsible for the rate of pulsation of the contractile vacuole until the rates of oxidation in *Paramecium* at corresponding temperatures have been determined. There is no doubt about the existence of three breaks in the lower plot of Fig. 1, indicating that pulsation depends upon a catenary series of at least three underlying processes. Which one of these will be the determining reaction depends upon the temperature range considered.

SUMMARY.

1. The rate of pulsation of the anterior contractile vacuole of *Paramecium caudatum* under chloretone anesthesia has been determined over a range of temperatures from 9–31°C. It has been found that the rate is a logarithmic function of the temperature according to the Arrhenius equation. From 9–16° the temperature characteristic (μ) has the value 25,600; from 16–22° it is 18,900; and from 22–31° it becomes 8,600.

2. It is concluded that there are at least three underlying reactions responsible for pulsation, the rates of which vary. Which reaction becomes the limiting one depends upon the range of temperature considered.

3. It does not appear that oxidative processes alone determine the rate of pulsation, although they may be of fundamental importance.

BIBLIOGRAPHY.

Cole, W. H., and Richmond, E., 1924–25, *Proc. Soc. Exp. Biol. and Med.*, xxii, 231.

Crozier, W. J., 1924–25, *a*, *J. Gen. Physiol.*, vii, 123; 1924–25, *b*, *J. Gen. Physiol.*, vii, 189.

Crozier, W. J., and Federighi, H., 1924–25, *J. Gen. Physiol.*, vii, 137.

Crozier, W. J., and Stier, T. B., 1924–25, *a*, *J. Gen. Physiol.*, vii, 429; 1924-25, *b*, *J. Gen. Physiol.*, vii, 571.

Glaser, O., 1924–25, *J. Gen. Physiol.*, vii, 177.

Kanitz, A., 1907, *Biol. Centr.*, xxvii, 11.

Khainsky, A., 1911, *Arch. Protistenk.*, xxi, 1.

Loeb, J., and Chamberlain, M. M., 1915, *J. Exp. Zool.*, xix, 559.

THE MECHANISM OF CHANGE IN RESISTANCE OF ERYTHROCYTES TO HYPOTONIC SALT SOLUTIONS.*

By S. C. BROOKS.

(From the Division of Pharmacology, Hygienic Laboratory, Washington, D. C.)

(Accepted for publication, February 17, 1925.)

A series of interesting papers has been published recently by Ashby[1] in which she presents data tending to show that the alkali metal cations may be grouped into two classes, according to their tendency to increase or to decrease the resistance of erythrocytes to hypotonic hemolysis in NaCl solutions. $K^{\cdot}$ and $Rb^{\cdot}$ decrease the resistance of human red blood cells, which contain $K^{\cdot}$ in large amounts, while $Na^{\cdot}$ and $Li^{\cdot}$ increase it; but when the studies were extended to cells which contain practically no $K^{\cdot}$ these effects were reversed (dog, ox, cat).

These experiments have been repeated, in part, with the addition of parallel series in which the fragility of the cells was determined in dilute KCl solution, in order to test the possibility that a reciprocal relation existed between the ions used to produce and to test the changes in fragility. The results fail to substantiate Ashby's results in certain important respects and are given here in the hope that others will be induced to repeat the experiments to confirm or disprove the points thus brought into question.

PROCEDURE.

Ashby added 5 to 10 cc. of citrated or defibrinated blood to 100 cc. of a 0.154 N NaCl solution or to isotonic KCl, LiCl, or RbCl solutions, and incubated the mixture at 36°C. for from 1 to 3 days, shaking every 24 hours. Asepsis was maintained. The cells were then collected by centrifugation and their fragility determined by observations on the

* Approved for publication by the Surgeon General, United States Public Health Service.

[1] Ashby, W., *Am. J. Physiol.*, 1924, lxviii, 239, 250, 585, 611.

degree of hemolysis in sodium chloride solutions of various concentrations to which were added in each case sodium citrate in quantity sufficient to be isotonic with 0.06 per cent NaCl; 0.02 cc. of cell sediment was added to each 1.0 cc. portion of the test solution.

The use of citrate in these experiments seems objectionable because of the introduction of an additional ion whose effects ought to be determined separately, and since it is not indispensable our experiments were conducted with defibrinated blood only; the sodium citrate was left out of the hypotonic test solutions. In some of the experiments these test solutions were made up with KCl as well as with NaCl. The effects of $Li^{\cdot}$ and $Rb^{\cdot}$ were not investigated.

Extreme care was used in the choice of materials. The distilled water was distilled once from block tin, and again from a well used Pyrex glass still in which the water and its vapor touched nothing else but well steamed out glass wool and absorbent cotton; its specific conductivity when fresh distilled was of the order 5×10^{-6} ohms^{-1}. Only Pyrex glass was used, except momentarily in the case of volumetric flasks and pipettes. Five samples of salts were used: pre-war Kahlbaum's NaCl and KCl; Squibb's reagent NaCl purporting to contain no potassium, and less than 0.0039 per cent of bi- and trivalent bases; a sample of KCl several times recrystallized for the preparation of calomel half-cells; and NaCl repurified by precipitation by HCl and subsequent heating in a stream of nitrogen.[2] No differences in results were traceable to differences in the samples used. Asepsis was maintained until the moment of testing for hypotonic hemolysis. Just prior to this the suspensions of red blood cells were tested for the presence or absence of intercurrent bacterial growth by plating out measured samples on nutrient agar.

The solutions used for incubation were sterilized in Pyrex glass and their subsequent pH found to be 6.6 to 6.9, showing the presence of only minimal traces of basic materials dissolved from the glass. The solutions in which the cells were incubated were therefore of a very high degree of purity and possessed so little buffering power as to have no influence on the pH during incubation; the addition of sodium citrate for buffering purposes was therefore unnecessary. The stock

[2] The last two were furnished by the Division of Chemistry, Hygienic Laboratory.

solutions were made up to be accurate within ± 0.1 per cent of their concentration, proper correction being made for the temperature of the water at the time of diluting to the final volume. These stock solutions, in which the cells were incubated, and by dilution of which the hypotonic test solutions were prepared, were 0.900 per cent NaCl and 1.150 per cent KCl (1.140 per cent in the last two experiments—this is more exactly isotonic with 0.9 per cent NaCl). The hypotonic solutions were prepared in 20 or 40 cc. lots with an error of ± 0.002 per cent in the case of NaCl and the same proportional error in the case of KCl.

Blood from six species of animals was used: sheep and dog, whose red blood cells contain little or no K^+; and horse, guinea pig, rabbit, and man, whose erythrocytes are rich in K^+. The blood was obtained from the jugular vein (sheep and horse), heart, (dog, guinea pig, and rabbit, the last under light ether anesthesia), and median basilic vein (human). No effect of the ether anesthesia was observable in the case of rabbit blood.

Portions of blood were poured into ten times their volume of either NaCl 0.9 per cent, or KCl 1.15 or 1.14 per cent (both sterile) and incubated at 37.5°C. for 1 or 2 days. Longer incubation was impossible because of excessive stromatolysis.

The normal resistance to hypotonic hemolysis was determined by tests on unwashed cells or cells washed once in ten volumes of a balanced salt solution isotonic with 0.9 per cent NaCl. Except as noted in one experiment this contained NaCl, KCl, $CaCl_2$, and $MgCl_2$ in the proportions customary for Ringer-Tyrode solution. In all cases the normal cells were gathered into a small volume by centrifugation and decantation of the supernatant fluid (serum or salt solution) and tested for fragility by adding 0.05 cc. to 2.0 or 2.5 cc. of each of the hypotonic solutions.[3] The blood incubated in the sodium and potassium chloride solutions was treated in exactly the same way after incubation. The cells were resuspended once by shaking after the first

[3] The error introduced by the higher osmotic pressure of the suspending fluid added with the cells to the hypotonic test solutions is nowhere greater than one-half of the interval between adjacent solutions, and in each experiment it is always the same in any given test solution; it may therefore be neglected by comparison with other errors.

24 hours of incubation. Further details of technique are given under the individual experiments.

RESULTS.

The experimental results are given in two different forms. The first is that used by Ashby, in which the degree of hemolysis in each

TABLE I.

Hemolysis in Hypotonic Solutions of Cells Incubated in Isotonic KCl and NaCl Solutions.

Red blood cells from	Incubated in	Tested in	Degree of hemolysis in NaCl solutions of the concentrations given in per cent, and in KCl solutions isotonic with them.													
			0.270	0.315	0.360	0.405	0.450	0.495	0.540	0.585	0.630	0.675	0.720	0.765	0.810	0.855
Rabbit (Experiment 1).	Control....	KCl	4	4–	2	1	0	0	–	–	–	–	–	–	–	–
		NaCl	4	4	3	1	0	0	–	–	–	–	–	–	–	–
	KCl.....	KCl	4	4	4	4	4	4	3+	–	–	–	–	–	–	–
		NaCl	4	4	4	4	4	4	4	–	–	–	–	–	–	–
	NaCl.... ..	KCl	4	4	4	4	3	1	0	–	–	–	–	–	–	–
		NaCl	4	4	3+	2+	2	1	0	–	–	–	–	–	–	–
Sheep (Experiment 3).	Control....	KCl	–	–	–	–	4	4	4–	3–	0	0	0	0	–	–
		NaCl	–	–	–	–	4	4	4–	2	1+	0	0	0	–	–
	KCl.....	KCl	4	4	4	4	4	4	4	4	4	4	4	4	3+	2
		NaCl	4	4	4	4	4	4	4	4	4	4	3+	3–	2	0
	NaCl.....	KCl	4	4	4	4	4	4	4	4	3+	2	1	0	0	0
		NaCl	4	4	4	4	4	4	4	4–	2	1	0	0	0	0

Control cells washed in a solution containing NaCl, KCl, $CaCl_2$, and $MgCl_2$ in the proportions usual for Ringer-Tyrode solution, and tested immediately, without incubation.

The degree of hemolysis is indicated by the figures 0, 1, 2, 3, 4, ranging from *no hemolysis* to *complete hemolysis* as determined by macroscopic observation of the turbidity.

test solution is given, the figures 0–4 representing increasing degrees of hemolysis from that too small to detect up to complete hemolysis. In my experiments, and presumably in Ashby's also, the degree of hemolysis was estimated by macroscopic observation of the turbidity

of the test suspensions (Tables I and II). In the last two experiments (Table III and Figs. 1 to 4) the degree of hemolysis was accurately determined: the intact cells were separated by centrifugation and decantation, hemolyzed in a volume of distilled water equal to that of the decanted supernatant fluid, and the comparative color of decantate and hemolyzed sediment used as a basis for calculating the proportion of the cells hemolyzed by the hypotonic test solution. These figures,

TABLE II.

Hemolysis in Hypotonic Solutions of Cells Incubated in Isotonic KCl and NaCl Solutions. Experiment 4.

Red blood cells from	Incubated in	Tested in	Degree of hemolysis in NaCl solutions of the concentrations given in per cent, and in KCl solutions isotonic with them.																
			0.18	0.225	0.27	0.315	0.36	0.405	0.45	0.495	0.54	0.585	0.63	0.675	0.72	0.765	0.81	0.855	0.90
Man.	Control...	KCl	4	3−	2−	1	0	0	0	0	0	−	−	−	−	−	−	−	−
		NaCl	4	3	2−	Tr.	0	0	0	0	0	−	−	−	−	−	−	−	−
	KCl.....	KCl	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	2
		NaCl	4	4	4	4	4	4	4	4	4	4	4	4	3+	2+	0	0	0
	NaCl.....	KCl	−	−	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		NaCl	−	−	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sheep.	Control...	KCl	−	−	−	4	4	4	3	1+	0	0	0	−	−	−	−	−	−
		NaCl	−	−	−	4	4	4	3+	1+	0	0	0	−	−	−	−	−	−
	KCl.....	KCl	4	4	4	4	4	4	4	4	4	4	4−	3	1	0	0	0	0
		NaCl	4	4	4	4	4	4	4	4	4	4	4−	3	1−	0	0	0	0
	NaCl....	KCl	−	−	4	4	4	4	4	3	2	1	0	0	0	0	0	0	0
		NaCl	−	−	4	4	4	4	4	3+	2+	1−	0	0	0	0	0	0	0

Controls and legend as in Table I.

in per cent, are properly corrected and plotted against the concentration of the test solutions as abscissæ (Figs. 1 to 4). The resistance of the cells is considered to be inversely proportional to the concentration of NaCl in which a given degree of hemolysis occurs; the mean of the values for 20, 40, 60, and 80 per cent hemolysis is here used.[4] The

[4] The details and advantages of this method of titration are given in Brooks, S. C., *J. Med. Research*, 1919–20, xli, 399.

theoretical significance of resistance as determined in this way is perhaps dubious, but it is still at least a concrete measure of the phenomena in which we are here interested, and as such it will be used.

Experiments 1, 3, and 4 (Tables I and II) show clearly that perfectly pure KCl solutions acting on cells not at any time exposed to such extraneous substances as sodium citrate, is uniformly injurious[5] regardless of whether the cells contain much or little potassium. NaCl, under similar conditions has little effect, either injurious or otherwise; an increase in the resistance of human cells might possibly be deduced from Table II. This will be referred to below.

These first experiments fail almost completely to show the reversal of the effects of $Na^{\cdot}$ and $K^{\cdot}$ which Ashby's experiments would lead us to expect when they act upon one of the two types of cells ($K^{\cdot}$-rich and $K^{\cdot}$-poor) instead of the other. But in these experiments the normal cells were washed once in a balanced salt solution. The effects of washing the cells are inadequately considered in Ashby's work and hence direct comparison would be unfair, especially as she does not tell how her control cells were treated. If washing in the modified Ringer-Tyrode solution increased the resistance, as might be expected from the work of Brinkman and van Dam,[6] then any increase in resistance caused by NaCl would be compensated for by a similar increase in the case of the washed control cells, and would thereby be concealed.

For this reason further experiments were done, in which the effects of washing in 0.9 per cent NaCl and in balanced salt solutions isotonic with 0.9 per cent NaCl were compared with the effects of incubation in the NaCl and KCl solutions. Ashby's method of evaluating resistance to hemolysis was so unsatisfactory for quantitative purposes that the precise method of hemolytic titration devised by the writer[4] was adapted to the present purpose as already outlined. Unwashed cells were used as controls, and all cells were suspended in just enough additional supernatant fluid to make them easy to handle, the final volume of the stock suspension of cells being only 15 to 25 per cent greater than that of the cell sediment obtained by centrifugation. In the first four experiments, each lot of cells had been suspended in a

[5] The term "injurious" is here to be understood as meaning only that the resistance of the red blood cells to hypotonic hemolysis is decreased.

[6] Brinkman, R., and van Dam, E., *Biochem. Z.*, 1920, cviii, 35.

volume equal to that of the blood from which it had been derived; when considerable hemolysis had occurred during incubation, correspondingly fewer cells were added to each test solution. This error

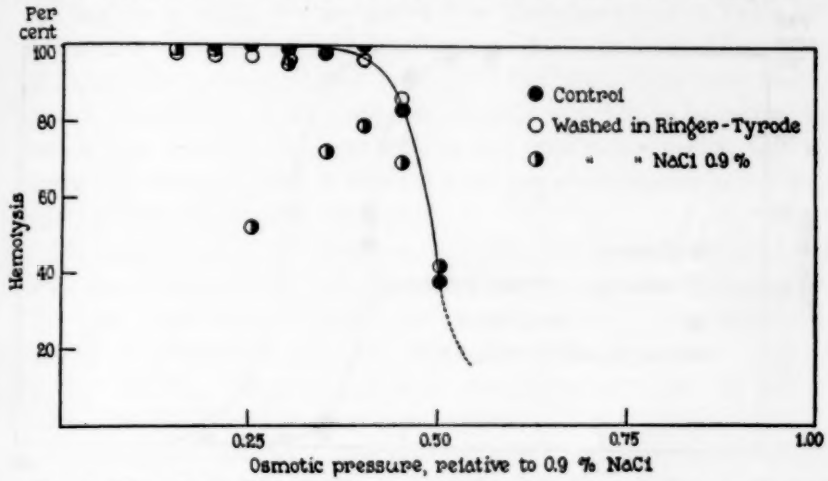

FIG. 1. The effect of different preceding treatments of dog erythrocytes on the degree of hemolysis (ordinates) produced by various dilutions of a 0.9 per cent NaCl solution (abscissæ). (Experiment 5.)

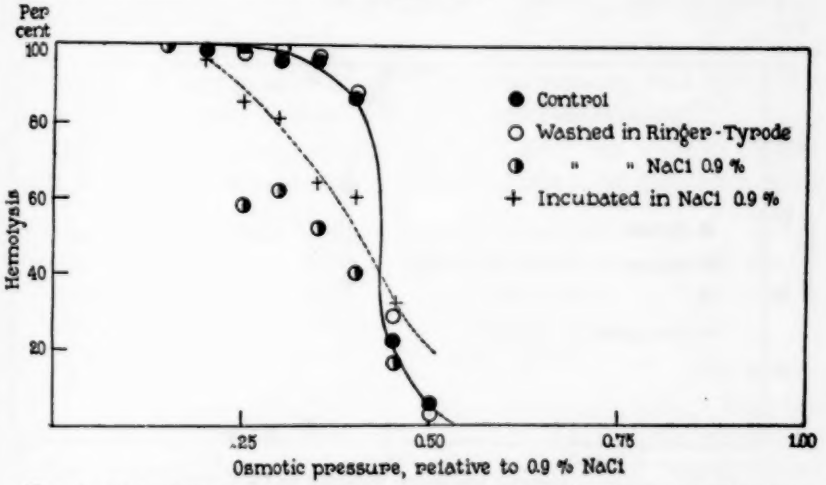

FIG. 2. The effect of different preceding treatments of guinea pig erythrocytes on the degree of hemolysis (ordinates) produced by various dilutions of a 0.9 per cent NaCl solution (abscissæ). (Experiment 5.)

was therefore eliminated by the change in procedure, and in addition the protective effects of the salts or serum constituents carried in with the supernatant fluid of the cell suspension were considerably reduced,

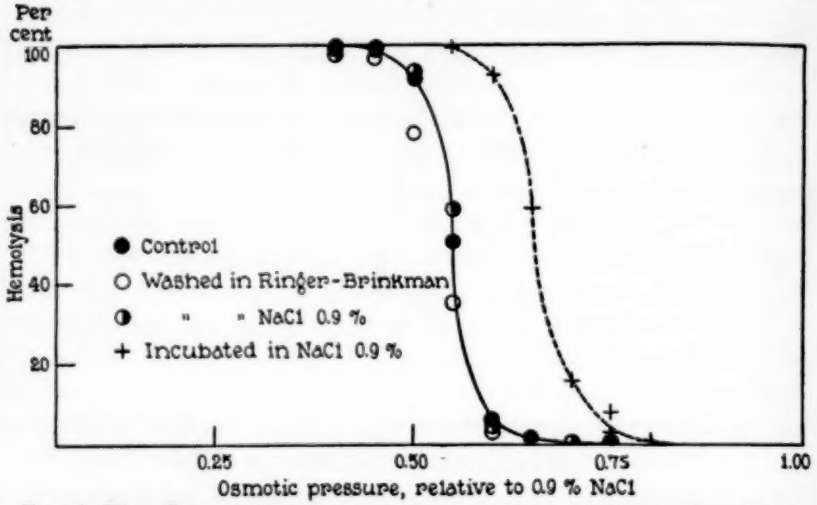

FIG. 3. The effect of different preceding treatments of dog erythrocytes on the degree of hemolysis (ordinates) produced by various dilutions of a 0.9 per cent NaCl solution (abscissæ). (Experiment 6.)

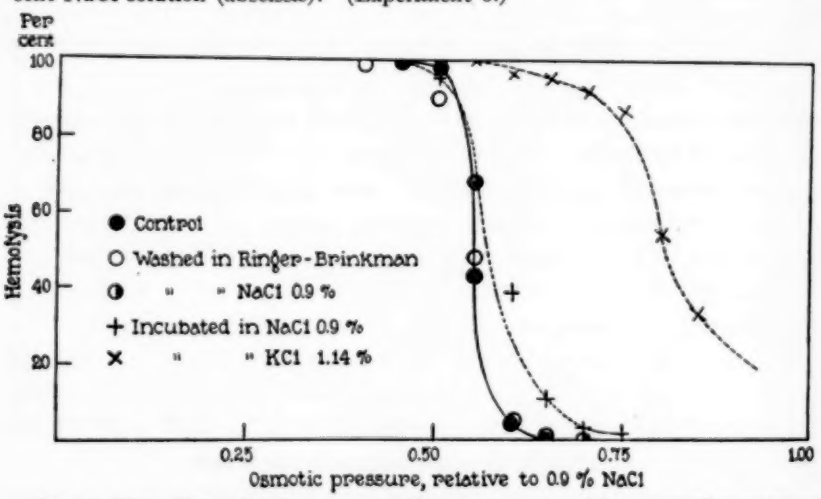

FIG. 4. The effect of different preceding treatments of horse erythrocytes on the degree of hemolysis (ordinates) produced by various dilutions of a 0.9 per cent NaCl solution (abscissæ). (Experiment 6.)

as was necessary to keep pace with the greater precision of the titration method.

Figs. 1 to 4 are the actual titration curves of these experiments, and the degrees of resistance calculated from them are given in Table III. In the first of the two experiments thus presented the incubation period of 2 days was so long that all but one of the lots of cells were nearly or quite completely hemolyzed, and no cells were left to be tested for resistance. In the other experiment the incubation period was reduced to 1 day, but even in this time the dog erythrocytes in the KCl solution were completely hemolyzed.

The error in these titrations is probably not much less than 3 per cent, and differences less than that may, for our purposes, be neglected. The error might have been greatly reduced by doing the tests in duplicate or triplicate, but this did not seem worth while since Ashby's limit of accuracy was from 5 to 15 or even 50 per cent, and the differences which she observed were even greater.

Washing the cells in a balanced solution has no material effect, whether modified Ringer-Tyrode solution or the solution employed by Brinkman and van Dam[6] be used, as in Experiment 6.[7]

Washing in NaCl 0.9 per cent had considerable effect in one case, that of guinea pig blood. The resistance was increased about 20 per cent, the irregularity of the titration curve making the uncertainty of this figure very great. In all other cases washing with NaCl solution had effects smaller than the experimental uncertainty.

The effects of incubation in pure salt solutions were as follows: potassium chloride led in all cases to a decrease in resistance, while

[7] The exact composition of this solution was as follows:

$NaCl$	7.60 gm.
$NaHCO_3$	1.70 "
KCl	0.20 "
$CaCl_2, 2H_2O$	0.133 "
H_2O *q. s.*	1,000 cc.

This may for convenience be called Ringer-Brinkman solution. These proportions of $CaCl_2$ and $NaHCO_3$ are supposed to determine the hydrion and calcion concentrations, as 0.45×10^{-7} N and 0.75×10^{-3} N respectively, but the solution here used had a pH of 8.35, ($H^{\cdot} = 0.45 \times 10^{-8}$), and ($Ca^{\cdot\cdot}$) was presumably about 0.075×10^{-4} N. The $NaHCO_3$ used was found to have contained more than the declared amount of Na_2CO_3.

sodium chloride decreased the resistance of dog blood markedly, that of horse blood by an amount hardly greater than the experimental error, and increased that of guinea pig blood by an amount considerably less than that due to washing in the same solution.

The observed amounts of hemolysis or hemochromolysis agreed with the measures of the volumes of intact cells and ghosts after incubation, and with the effects of incubation on resistance to hemolysis, all of them showing that injury was greatest when $K^{\cdot}$-poor cells were exposed to KCl, and less in the order: $K^{\cdot}$-poor, with NaCl; $K^{\cdot}$-rich, with KCl; $K^{\cdot}$-rich, with NaCl. Reduction of oxyhemoglobin was marked in the first of these combinations and decreased in the same order as the degree of injury; this fact suggests that autoxidation processes were accelerated when injury occurred. A cause and effect relation might conceivably be present, but it seems more likely that the phenomena are end-results of common causes.

DISCUSSION.

The results of my experiments are in conflict with Ashby's in that there is no case in which a clear-cut increase in resistance to hemolysis can be attributed to incubation of red blood cells of either type, $K^{\cdot}$-rich or $K^{\cdot}$-poor, in either NaCl or KCl solutions. The one case in which any increase of resistance is recorded, namely that of guinea pig blood in 0.9 per cent NaCl, is based on a very uncertain titration curve, and may well be meaningless. Accurately determined titration curves of other blood cells of the $K^{\cdot}$-rich group show no such effect. It must be noted, however, that merely washing another portion of these same guinea pig cells in 0.9 per cent NaCl was in itself enough to cause an apparent increase in resistance, which much exceeded that found after incubation in the same solution. Evidently the resistance decreased during incubation, and merely appeared to be increased because, due to the effect of washing, it started high; or else, possibly exposure to pure solutions of NaCl first increases and later decreases the resistance of red blood cells like those of the guinea pig.

The results of my experiments also conflict with Ashby's in that, irrespective of the type of cell, KCl was always more injurious than NaCl. The conflict of results may be due to impurity of materials

used by Ashby, to the use, by her, of such extraneous substances as sodium citrate, to mechanical injury of the cells during defibrination in the case of the present experiments, or to the fact that we have studied different phases of time processes which produce opposite effects according to the stage at which they are interrupted.

Mechanical injury due to defibrination as used in these experiments may be discounted since the serum of the blood used was found to be but slightly discolored by hemoglobin even after the blood had been standing at 3–25°C. for the duration of the experiment.

The presence of traces of citrate or of bivalent metals dissolved from glassware and carried into the incubation mixtures in Ashby's experiments may have so far antagonized the effects of really pure KCl or NaCl as to mask their real effects. If so, Ashby's conclusions do not concern $K^{\cdot}$ and $Na^{\cdot}$, but unknown mixtures of salts in partially balanced solutions.

The study of the last possibility requires further experiments with solutions of known purity in which the blood cells, free from substances foreign to blood, are incubated for various periods of time and their resistance to hypotonic hemolysis then determined by precise titration. In view of the unknown influence exerted by serum constituents it is doubtful whether more profitable lines of study might not be found. Enough has been said to demonstrate that Ashby's work requires much substantiation before we may regard it as proven that the effects of $Na^{\cdot}$ and $K^{\cdot}$ on red blood cells are opposite and that the sense of their effects is reversed according to whether the erythrocytes used are $K^{\cdot}$-rich or $K^{\cdot}$-poor. With this, her conclusion that the effects of $K^{\cdot}$ and $Na^{\cdot}$ are due wholly to the nature of the cells upon which they act falls to the ground also.

SUMMARY.

Ashby's work on the effects of KCl and NaCl on the resistance to hypotonic hemolysis of $K^{\cdot}$-rich and $K^{\cdot}$-poor erythrocytes has been repeated with great attention to purity of materials and refinement of technique. The results fail to agree with those of Ashby.

1. KCl produces greater loss in resistance to hypotonic hemolysis than does NaCl, irrespective of the species of the animal from which the cells are taken.

2. While cases of an increase in resistance have been encountered in my experiments, they are either very slight, or else the particular determination is subject to very great uncertainty. The great increases in resistance found by Ashby are not even approached in any of the present series of experiments.

3. Ashby's generalization that KCl and NaCl have opposite effects on red blood cells, and that the sense of these effects depends on whether the cell is $K^{\cdot}$-rich or $K^{\cdot}$-poor is not substantiated.

HYDROGEN ION CONCENTRATIONS IN THE BLOOD OF INSECTS.

By R. W. GLASER.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, March 4, 1925.)

Most investigations on the acidity of blood, lymph, and tissue fluid have dealt largely with higher animals. However, three papers have recently appeared which deal partly with the hydrogen ion concentrations in the blood of insects. Jameson and Atkins (1921) reported that the blood of the silkworm gave the pH value 6.8. Bishop (1923–24) averaged determinations with the blood of honey bee larvæ and found the pH to be 6.83, ranging from 6.77 to 6.93. During spinning an acidosis of the blood appears, giving a range of pH value from 6.60 to 6.65. After spinning the pH returns to approximately the initial level. Among other interesting things, Bishop states, "The acidosis that occurs during spinning appears to be one of several factors present that should encourage autolytic changes in a tissue. Such changes do, in fact, take place in the bee larva. High concentration of the H ion may, therefore, be assigned as a contributing cause to the rapid metamorphosis of this insect." Bodine (1925) in Orthoptera found the average blood pH value for seventeen species of grasshoppers to be 6.8.

In connection with some other work it became necessary for the writer to make frequent pH determinations of the blood of a variety of insects. The colorimetric method described by Brown (1923–24) was employed.

The blood is obtained from the insects by rapidly cutting off a leg or proleg, or by inserting a fine needle in a favorable locality. A standard platinum loopful of the blood is obtained as it oozes forth, and the determinations then made according to the method of Brown. Since each determination can be executed in less than 1 minute, collec-

tion of the blood under oil may be omitted. Brom-thymol blue was the indicator finally adopted after checking with various indicators recommended by Clark (1920). This indicator is the one which was also used by Crozier (1923–24) in his observations on the reaction in

TABLE I.

Species.	Metamorphosis.	No. tested.	Age.	Range of pH of blood at different ages.	Range of pH of blood for entire cycle.
Melanoplus differentialis.	None.	10	Young.	7.4–7.6	7.2–7.6
" "	"	10	Adult.	7.2–7.6	
Periplaneta americana.	"	5	Young.	7.5–8.0	7.5–8.0
" "	"	5	Adult.	7.5–8.0	
Malacosoma americanum.	Complete.	10	3rd stage.	6.6–6.8	6.4–7.4
" "	"	10	4th "	6.6–6.8	
" "	"	10	Last "	6.4–7.2	
" "	"	10	Spinning "	6.4–7.0	
" "	"	10	Pupal "	6.6–7.2	
" "	"	10	Moth "	6.8–7.4	
Bombyx mori.	"	6	2nd "	6.8–7.2	6.4–7.2
" "	"	6	3rd "	6.6–7.1	
" "	"	6	4th "	6.6–7.2	
" "	"	6	Last "	6.6–7.0	
" "	"	6	Spinning "	6.6–7.0	
" "	"	6	Pupal "	7.0	
" "	"	6	Moth "	6.4–7.2	
Musca domestica.	"	6	Maggot "	7.4–7.6	7.2–7.6
"	"	6	Pupal "	7.4–7.6	
"	"	6	Fly "	7.2–7.6	

different parts of the gut of Psychoda and Chironomus. In a footnote Crozier points out that, "The numerical pH values obtained with this indicator may be subject to 'salt correction' of unknown but probably quite small magnitude."

The results of the present determinations are summarized in Table I. Two insects which have no metamorphosis were first chosen, namely a species of grasshopper and the large American roach. The third

column gives the number of animals used; the fourth, the approximate age; the fifth, the range of the pH readings at different ages; and the last column, the range covering most of the life cycle of the forms used. Later, three insects (the American tent caterpillar, the silkworm, and the house-fly) having a complete metamorphosis were chosen. Determinations on these forms were begun as soon as they were old enough to yield a sufficient amount of blood and were continued through to maturity. In the cases of the tent caterpillar and silkworm, a stage has been inserted between the last larval and the pupal stage. This has been tentatively labeled the "spinning stage," and was studied in order to bring out any pronounced decrease or increase in pH which might occur before, during, or after metamorphosis.

No great differences in pH exist between young and mature grasshoppers. The range is 7.2 to 7.6. The same was noticed in the case of young and mature roaches; the range is 7.5 to 8.0. House-fly maggots, pupæ, and adults have a range that does not vary much with the stage; and the entire range, 7.2 to 7.6, agrees with that for the grasshoppers. In this case, then, no definite increase or decrease in hydrogen ion concentration appears to accompany metamorphosis. The blood of the two lepidopterous species shows, in general, a greater degree of acidity than was the case with the last three forms. The entire range for *Malacosoma americanum* is 6.4 to 7.4, and the entire range for *Bombyx mori* is 6.4 to 7.2. In these two species also the ranges are such that one could not possibly correlate decrease or increase in pH with metamorphosis. A point that is emphasized is the stability of the silkworm pupal reaction as contrasted with the pupal reactions of the tent caterpillar and house-fly. The writer hesitates to offer an explanation for this fact.

Why does the blood of animals of the same age or stage give such divergent pH readings? Certainly the difference between 6.4 and 7.2 is not slight. The question is not easily answered, for up to the present little is known concerning metabolism in insects. Differences in food occur which might explain the matter. However, silkworms eat only mulberry leaves; the moth eats nothing, and yet the pH of the body fluids of moths ranges between 6.4 and 7.2. The writer is inclined to believe that these pH variations are expressions of

inherent differences and cannot be explained on a simple metabolic basis.[1]

As regards the view that decrease or increase in pH may be correlated with metamorphosis, there seems to be no doubt that the results obtained with bees (Bishop) are correct; but evidently it would be extremely unsafe to generalize as to the mechanism of metamorphosis from this standpoint. In this connection it might be well to quote Dernby (1918). "In what sense the hydrogen ion concentration will change during autolysis is impossible to predict. This depends upon the chemical constituents of different tissues and might vary in any direction."

SUMMARY.

The range of pH values for the blood of grasshoppers and of houseflies is 7.2 to 7.6. The range of values for roaches is 7.5 to 8.0. The range for *Malacosoma americanum* is 6.4 to 7.4; and the range for *Bombyx mori* is 6.4 to 7.2. From the work of other investigators and from the writer's results, it is apparent that the pH of insect blood, in general, may vary between 6.4 and 8.0. In the forms observed no correlation exists between blood pH and age, nor between pH and metamorphosis.

BIBLIOGRAPHY.

Bishop, G. H., 1923–24, *J. Biol. Chem.*, lviii, 543.
Bodine, J. H., 1925, *Biol. Bull.*, xlviii, 79.
Brown, J. H., 1923–24, *J. Lab. and Clin. Med.*, ix, 239.
Clark, W. M., 1920, The determination of hydrogen ions, Baltimore.
Crozier, W. J., 1923–24, *J. Gen. Physiol.*, vi, 289.
Dernby, K. G., 1918, *J. Biol. Chem.*, xxxv, 179.
Jameson, A. P., and Atkins, W. R. G., 1921, *Biochem. J.*, xv, 209.

[1] The variations cannot be due to CO_2 from the air absorbed during the time when the determinations were made. Two or three determinations on the blood of an individual insect made on the same day always give the same reading, whereas determinations made on the blood of separate individuals may or may not give the same result. Anyway, insect tissue is aerobic, receiving air directly through tracheæ, so CO_2 tension within the animals must vary somewhat. Nevertheless, the pH determinations made represent conditions within and were not influenced much after the removal of the blood, however much they may be influenced by variations in CO_2 tension within. The explanation offered for the variations in pH may also be applied to variations in CO_2 tension if such variations exist within the insects.

A TEST FOR DIFFUSIBLE IONS.

II. The Ionic Nature of Pepsin.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, March 7, 1925.)

It was pointed out in a previous paper[1] that the distribution of a substance, when added to a system possessing the properties required for the establishment of a Donnan equilibrium, could be used as a test for the ionic nature of the substance. If the substance is a diffusible ion it must be distributed in such a way that the ratios of its activities in the two parts of the system are the same as the ratios of the activities for the other ions (of the same valence). Since in dilute solution the activities may be assumed approximately equal to the concentration, the concentration ratios may be used provided there is no unionized compound present in the solution. It has been found that the ratio of the concentration of pepsin inside of gelatin or egg albumin particles to the concentration outside is approximately equal to the ratio of the chloride or bromide ion under the same conditions. This is true over the range of pH from 1 to 7, and in the presence of various salts and acids. It follows, therefore, that pepsin is a monovalent anion, and also that the enzyme does not form a compound with gelatin nor is the degree of dissociation affected by changes in pH in the range in which the enzyme is active. The enzyme becomes very unstable on the alkaline side of pH 7, and it is, therefore, not possible to perform the experiments in alkaline solution.

Experimental Procedure.

Pepsin.—The pepsin solution used was prepared by dissolving 10 gm. of Park, Davis 1/10,000 pepsin in 100 cc. of water, titrating to pH 2 with nitric acid, and dialyzing against 0.01 N nitric acid for 3 days. The solution was then brought to pH 3.0 and filtered. The resulting solution is about ten times as

[1] Northrop, J. H., *J. Gen. Physiol.*, 1923–24, vi, 337.

active per gm. dry weight as the original. Several other pepsin preparations were used occasionally. No difference in the results was noted. In most cases 2 cc. of the pepsin solution were added to 50 cc. of the protein suspension.

Determination of Pepsin.—The relative quantity of pepsin was determined by noting the rate of change in the viscosity of a standard gelatin solution as described by Hussey and the writer[2] except that 0.2 cc. samples were used to 10 cc. of gelatin, and a small (2 cc.) viscometer was used. The standard gelatin solution used contained 1.3 per cent of gelatin and 0.066 M of phosphoric acid. The unit of pepsin is taken as that quantity which causes a 10 per cent decrease in 10 cc. of this standard gelatin solution in 1 hour at 34°C.

It was found that pepsin was removed from the solution by filtration so that it was necessary to make the determination on the supernatant fluid. The total amount of pepsin present was known from the control solution containing no protein and the amount of pepsin in the protein was then found by difference. In calculating the concentration in the gelatin or egg albumin no correction was made for the volume occupied by the protein itself, since it was found that at the isoelectric point the concentration of either pepsin or chloride was almost identical with that obtained when no protein was present.

The experimental procedure in general was the same as that described in the preceding paper. The protein particles were kept in the solution at 0°C. for 18 hours before adding the pepsin. The particles as a rule occupied 30 to 40 per cent of the total volume.

Since in these experiments only the total concentration of enzyme can be determined it is evident that the formation of any compound between the enzyme and the protein would destroy the agreement with the chloride ratios. It was noted in the case of trypsin that some such disturbing reaction occurred near the isoelectric point of gelatin. This irregularity was much more marked with pepsin and it soon became evident that there were two entirely distinct reactions occurring. Under certain conditions large amounts of the enzyme would be taken up by the gelatin and there was no correlation between the pepsin ratios and the chloride ratios. This was especially true at and near pH 4.7, the isoelectric point of gelatin. This adsorption of the enzyme was found to be very markedly affected by the electrolyte concentration. It does not occur in electrolyte-free solution nor when the salt

[2] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

TABLE I.

Effect of NaCl on Adsorption of Pepsin by Gelatin.

50 cc. of isoelectric gelatin suspension containing noted concentration of NaCl. 2 cc. of pepsin added and pepsin determined in supernatant liquid after 2 hours. 18 cc. of gelatin. 34 cc. of filtrate.

Concentration of NaCl.	Ratio: $\frac{\text{Pepsin conc. in gelatin}}{\text{Pepsin conc. in filtrate}}$ (chloride ratio = 1.0).
M	
0	1.0
0.0016	5.0
0.008	10.0
0.04	9.0
0.20	1.0
1.0	1.0

TABLE II.

Effect of Various Salts on Combination of Pepsin and Gelatin.

Concentration of all salts 0.01 N. Determinations made after 1 hr. at 0°C.

Salt.	Ratio: $\frac{\text{Pepsin in gelatin}}{\text{Pepsin in filtrate}}$.
NaCl	4.0
NH_4Cl	4.1
KCl	4.0
K_2SO_4	4.0
$CaCl_2$	5.0
$BaCl_2$	4.9

TABLE III.

Effect of Size of Particles and Concentration of Pepsin on Amount of Pepsin Removed.

2 gm. of sieved isoelectric gelatin of different sizes added to 50 cc. of M/100 acetate buffer pH 4.7, pepsin added, and suspension kept at 0°C. for 20 hrs. Gelatin filtered off, dissolved in water, and pepsin determined in 0.2 cc. of this solution. Total pepsin 200.

Relative total surface of gelatin.	Pepsin in gelatin.	Pepsin per unit surface.
3.5	140	40
2.0	100	50
1.0	45	45

concentration is more than 0.20 M. This result is shown in Table I. The chloride ratio is 1 in every case, but the pepsin in low concentrations of salt is nearly all in the gelatin. Table II shows that this effect is not markedly influenced by the character of the salt. This combination of pepsin and gelatin was found to be irreversible and not affected by the addition of inactive pepsin. It could be prevented by the addition of dissolved gelatin to the solution. It was further found

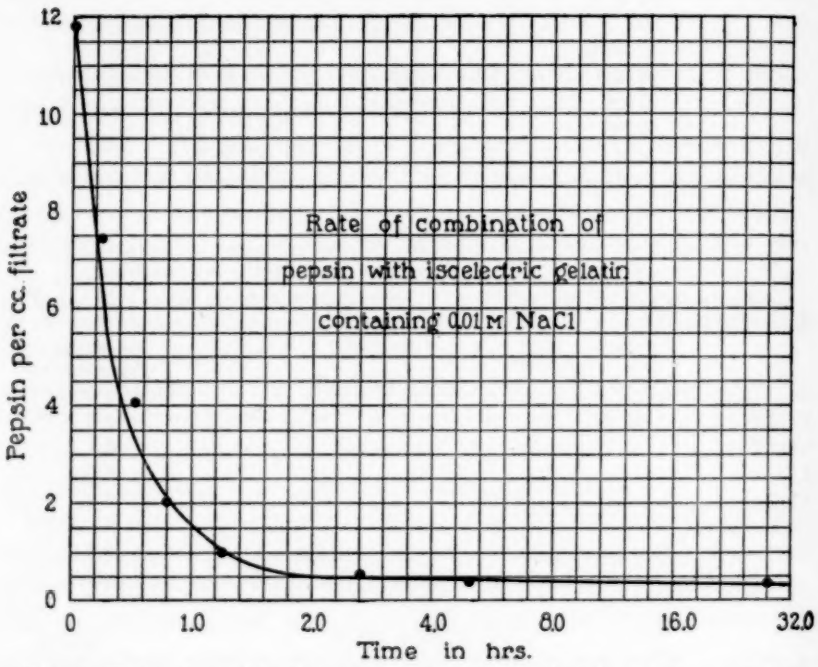

FIG. 1. Rate of combination of pepsin with isoelectric gelatin containing 0.01 M NaCl.

that under these conditions the amount of pepsin removed was directly proportional to the surface of the gelatin since small particles removed much more than large ones. Table III shows the results of an experiment in which the size of the particles was varied. The relative surface was determined roughly by measuring the diameter of a number of particles under the microscope. The results show that the amount of enzyme removed is almost directly proportional to the

surface of the gelatin particles. The writer found previously[3] that the removal of pepsin by coagulated egg albumin particles was independent of the surface. This experiment was repeated and confirmed in the course of the present work. This surface combination with gelatin particles differs also in that much larger amounts of enzyme are removed than is the case when the enzyme is distributed in accordance with the Donnan equilibrium. In Table III, for instance,

TABLE IV.

Time Required for Equilibrium with Gelatin.

50 cc. of gelatin suspension pH 4.7, 0.10 M KCl + 2 cc. of pepsin. Stirred at 0°C.

Time, *hrs.*	0	0.05	0.10	0.15	0.25
Pepsin per cc. filtrate	4.8 (calc.).	3.6	3.4	3.2	3.3

TABLE V.

Reversibility of Pepsin Distribution.

3 cc. of 0.1 N NaOH added to 25 cc. of gelatin suspension and the suspension kept at 0°C. for 18 hrs. 10 cc. of pepsin added and the solution stirred for 20 min., filtered, and the gelatin suspended in 50 cc. of 0.01 N KCl, stirred for ½ hr., and pepsin and chlorine determined in gelatin and in filtrate.

	a	*b*	*c*
Ratio: $\frac{\text{Pepsin conc. in gelatin}}{\text{Pepsin conc. in liquid}}$.	0.55	0.42	0.46
Ratio: $\frac{\text{Cl conc. in gelatin}}{\text{Cl conc. in liquid}}$.	0.65	0.64	0.60

nearly all the enzyme is removed by 2 gm. of gelatin, whereas only a few per cent can be accounted for on the basis of the chloride ratios.

The time required for this reaction is also much longer than that required when the amount of enzyme taken up by the protein agrees with the results calculated from the chloride ratios. This is shown in Fig. 1. There is a rapid decrease in the pepsin concentration of the supernatant liquid for the first 2 hours followed by a slow decrease which apparently is still taking place after 24 hours. When, however,

[3] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113.

sufficient electrolyte is present to prevent this adsorption reaction and cause the pepsin to be distributed by the ionic equilibrium, the solution comes to equilibrium very rapidly as may be seen from Table IV. This is not, of course, a true equilibrium with respect to time, since the gelatin would eventually be digested by the enzyme and the two phases would no longer exist. It is, however, a true value as long as the gelatin is not affected, since it can be reached from either side, as will be shown below.

It is evident from the above experiments that near the isoelectric point of gelatin and in the presence of the proper amount of electrolyte there is a secondary surface adsorption of the enzyme which entirely masks the distribution effect. This is also partially true on the acid side of pH 4.7, although the adsorption is less marked in this range. The fact that the maximum effect is obtained at the isoelectric point of the gelatin and that it is not noted with egg albumin renders it probable that the effect of the salt is on the gelatin rather than the pepsin.

On the alkaline side of pH 4.7 or in the presence of more than 0.2 M electrolytes the distribution of the pepsin in the system is the same as that of the chlorine ion and is reversible. In the preceding experiments the enzyme was added to the solution containing the gelatin particles so that the concentration of enzyme in the supernatant liquid first decreased and then remained constant. Table V, however, gives the results of an experiment in which the pepsin at the beginning was in the gelatin so that the concentration in the supernatant liquid increased. The results show that here also the pepsin and chlorine ratios are nearly equal, and therefore that the equilibrium may be reached from either side

Comparison of Chloride and Pepsin Ratios with Gelatin Particles on the Alkaline Side of pH 4.7.—The results of series of experiments with pepsin and various salt and alkali concentrations are given in Table VI. The ratios agree within the experimental error. The first five experiments are the averages of six separate determinations and are accompanied by their respective probable errors. The remaining results are based on single experiments and are therefore less accurate. The experiments cannot be carried out in more alkaline solutions owing to the rapid inactivation of the enzyme.

Experiments with Egg Albumin Particles.—It was found by Dauwe[4] that pepsin was removed from solution by coagulated proteins and it was shown by the writer[3] that the amount of enzyme removed in this way was a function of the pH of the solution, there being a maximum at pH 2.5 for egg albumin and at pH 3.5 for gelatin. It was suggested at that time that this combination of protein and enzyme was analogous to the combination of protein and inorganic ions and that it was due to the ionization of the protein. From the present point of view, however, it is evident that this explanation is not complete. Neither the

TABLE VI.

Comparison of Chloride and Pepsin Ratios with Gelatin at Various pH and Salt Concentrations.

pH	Salt.	Concentration of salt.	Ratio: $\frac{\text{Cl conc. in gelatin}}{\text{Cl conc. in filtrate}}$	Ratio: $\frac{\text{Pepsin conc. in gelatin}}{\text{Pepsin conc. in filtrate}}$
		M		
4.7	KCl	0.10	1.01 ± 0.01	1.01 ± 0.03
5.1	KCl	0.10	0.95 ± 0.01	1.06 ± 0.05
5.6	KCl	0.01	0.70 ± 0.01	0.61 ± 0.04
6.0	KCl	0.004	0.62 ± 0.02	0.51 ± 0.04
7.2	KCl	0.004	0.50 ± 0.013	0.51 ± 0.05
6.5	$NaNO_3$	–	0.65	0.53
	KCl	0.004		
6.5	$NaNO_3$	0.005	0.74	0.57
	KCl	0.004		
6.5	$NaNO_3$	0.040	0.88	0.80
	KCl	0.004		

inorganic ion nor the pepsin is really combined with the protein but are simply prevented from diffusing out of the protein particles by the electrostatic attraction of the non-diffusible protein ion. It is now clear why the maximum for digestion and for this combination did not coincide exactly. The maximum rate of digestion of the protein corresponds with the point at which it is most highly ionized, whereas the point at which the ratio of the concentrations inside and outside is a

[4] Dauwe, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 426.

maximum is in much less acid solution, owing to the depressing effect of the common ion of the acid on the equilibrium.

Influence of the Method of Preparation of the Egg Albumin.—It was found that egg albumin prepared simply by coagulation and washing, adsorbed the enzyme in the same way as isoelectric gelatin. If, however, the albumin was then dried for 24 hours at 70°C. this adsorption no longer occurred and the enzyme was distributed in the same way as the chloride ion. If the protein was allowed to stand several days in water, the adsorption reaction again became manifest. The adsorption is again apparently closely connected with the properties of the surface of the protein particles.

It is difficult to show directly that the distribution of ions in coagulated egg albumin particles is governed by the Donnan equation, since there is no way to determine the activity of the free ions in the protein, which in this case is a solid. The ratios of the total concentrations, as will be seen, have all the characteristics of a Donnan equilibrium, however, and it seems reasonable to assume that the mechanism is the same as in the case of gelatin. There is one striking difference in that the swelling of the albumin is not influenced by the pH nor the electrolyte concentration to nearly as great an extent as is the swelling of gelatin. This is no doubt due to the much greater resistance offered by the protein to the osmotic pressure. As a result of this characteristic the protein concentration in the particles is around 30 to 40 per cent, since 5 gm. of dry albumin usually occupied 12 to 15 cc. The values of the ratios, therefore, are very much higher than in the case of gelatin, since this value increases with the concentration of non-diffusible ion.

Reversibility of the Reaction.—Table VII gives the result of an experiment showing that the equilibrium point may be reached from either side. In the first part of the experiment the pepsin was added to a suspension of egg albumin in acid and then Na_2SO_4 added. This depresses the equilibrium and some of the pepsin diffuses out so that the concentration of enzyme in the supernatant liquid increases. If the sodium sulfate is added first, the enzyme is at first more concentrated in the supernatant liquid and diffuses into the egg albumin. The bromide behaves in the same way as the pepsin but more rapidly, as would be expected. The final ratios for the bromide and pepsin are

again approximately equal and are the same irrespective of the way in which the final value is obtained.

It was found that with egg albumin prepared as above about 1 hour was required for a constant value to be reached. The time varied somewhat with the conditions and the sample of egg albumin, so that it was necessary to run a preliminary experiment in which determinations were made at short intervals until no further change occurred. The remaining solutions were then analyzed at that time. As in the case of gelatin, this is not a permanent equilibrium point, since the albumin is slowly digesting. It would be expected from previous work that the pepsin would combine with the products of digestion of the

TABLE VII.

Equilibrium between Egg Albumin and Pepsin.

Solution 1: 5 gm. of egg albumin + 10 cc. of 0.1 N HBr. Total volume 50 cc. + 2 cc. of pepsin. Kept at 0°C. for 0.5 hr., and 5 cc. of M/2 Na_2SO_4 added.

Solution 2: 5 gm. of egg albumin + 10 cc. of 0.1 N HBr + 5 cc. of M/2 Na_2SO_4 made up to 55 cc. Kept at 0°C. for 18 hrs., and 2 cc. of pepsin added. Br and pepsin concentrations were determined in filtrate after time noted.

Time.	Concentration of Br.		Concentration of pepsin.		Ratio: $\frac{\text{Br in egg albumin}}{\text{Br in filtrate}}$.		Ratio: $\frac{\text{Pepsin in egg albumin}}{\text{Pepsin in filtrate}}$.	
	1	2	1	2	1	2	1	2
hrs.								
0.01	0.15	0.70	2.1	6.5	29		12	
0.20	0.70	0.70	3.85	3.9				
0.50	0.70	0.70	3.90	3.9	4.35	4.45	4.70	4.60
1.00	0.70	0.70	3.90	3.9				

protein and thereby become more concentrated inside the particles, and this is apparently the case since after several hours the concentration of pepsin in the solution decreases slowly.

The results of a number of experiments comparing the chloride and pepsin ratios under different conditions are shown in Tables VIII and IX. Table VIII shows that with a monovalent acid the ratio may reach very high values corresponding to a pH difference between the inside and outside of more than 1.0 and to a potential difference of over 70 millivolts. As was pointed out above, this is due to the high concentration of protein. The table also shows that the maximum

value of the ratio is near pH 3.0 instead of 4.2 as is the case with gelatin. This is the result expected from the titration curve of egg albumin as compared with gelatin, since the albumin is not completely combined with acid till a considerably higher hydrogen ion concentration is reached than is the case with gelatin. Owing to the high value of the ratio the results with HBr are inaccurate, since it was necessary to use a very large amount of pepsin in order to have a sufficient concentration in the supernatant liquid to determine. The table also shows the marked depressing effect of sulfate ion on the ratio.

TABLE VIII.

Comparison of Br or Cl and Pepsin Ratios at Various pH with H_2SO_4 and HCl.

Acid.	Concentration of acid.	pH	Ratio: $\frac{\text{Br in egg albumin}}{\text{Br in filtrate}}$	Ratio: $\frac{\text{Pepsin in egg albumin}}{\text{Pepsin in filtrate}}$
	M			
	–	6.0	0.70	0.71
HCl	0.004	4.5	4.6	4.1
"	0.008	4.2	10.0	7.2
"	0.020	3.8	23.0	15.0
"	0.04	3.3	36.0	22.0
H_2SO_4 + 0.02 KBr	0.002	5.0	2.5	2.0
" + 0.02 "	0.005	4.7	3.6	2.9
" + 0.02 "	0.010	4.4	4.3	4.6
" + 0.02 "	0.020	4.0	5.8	5.3
" + 0.02 "	0.040	2.8	7.8	7.7
" + 0.02 "	0.060	1.6	5.6	5.9
" + 0.02 "	0.100	1.0	4.2	4.6

Table IX gives the results of experiments with various salt concentrations on the alkaline and acid side. It will be noted that on the alkaline side Na_2SO_4 and $NaNO_3$ behave alike and $Ca(NO_3)_2$ depresses more powerfully, while on the acid side Na and Ca behave the same and SO_4 depresses. This is qualitatively the expected result since the value of the equilibrium ratio is governed almost entirely by the ion of the opposite sign of charge from that of the protein ion.

The foregoing experiments show that pepsin distributes itself quantitatively in the same way as does the chloride or bromide ion. This is the result predicted by Donnan's equilibrium theory if pepsin is itself

a negative monovalent ion. As far as the writer is aware there is no other theory which would predict such a result even qualitatively. If this view of the mechanism is correct, it follows that the enzyme is not actually combined with the protein at all and hence anomalies in the

TABLE IX.

Effect of Salts at pH 6.0 and 4.4.

pH 6.0: 0.004 M KCl + noted concentrations of salts.
pH 4.4: 0.01 M HBr + noted concentrations of salts.

Salt.	Concentration of salt.	Ratio: $\frac{\text{Cl in egg albumin}}{\text{Cl in filtrate}}$.	Ratio: $\frac{\text{Pepsin in egg albumin}}{\text{Pepsin in filtrate}}$
	At pH 6.0.		
	M		
$NaNO_3$	0	0.61	0.59
"	0.01	0.64	0.71
"	0.04	0.70	1.0
"	0.16	0.80	1.0
Na_2SO_4	0	0.67	0.55
"	0.005	0.78	0.80
"	0.02	0.90	0.90
$Ca(NO_3)_2$	0	0.63	0.55
"	0.0006	0.68	0.73
"	0.0025	0.85	0.78
"	0.010	1.0	1.0
	At pH 4.4.		
		Br ratio.	
$NaNO_3$	–	13.0	14.0
"	0.006	4.7	4.9
"	0.025	1.8	2.5
"	0.10	1.0	1.0
Na_2SO_4	0.003	3.9	3.0
"	0.012	2.0	2.0
"	0.05	1.1	1.0
$Ca(NO_3)_2$	0.006	3.0	3.0
"	0.025	1.70	3.0
"	0.10	1.0	1.3

kinetics of the digestion cannot be accounted for on the basis of such a compound formation. The results also affect experiments such as those of Arrhenius on the rate of diffusion of enzymes into gelatin. Unless conditions were chosen so that the final concentrations inside and outside were equal, the rate of diffusion would be markedly affected, and calculations based on the assumption that the final equilibrium would be reached when the enzymes were uniformly distributed throughout the system would be incorrect.

It has been frequently suggested that protein digestion is determined by the swelling of the protein. It may be seen from the present experiments that the more swelling the higher would be the relative enzyme concentration in the protein, since both the swelling and the distribution of the enzyme are determined by the amount of ionized protein (other factors being constant). There is no causal connection between the two, however, and the increased rate of digestion is not due to the hydration of the protein but simply to the increased concentration of the enzyme in the protein. The digestion of insoluble proteins is therefore a much more complicated problem than that of a protein in solution since the ionization of the protein, its concentration, and the concentration of the enzyme inside the mass of protein are all affected both by the pH and by the salt concentration.

SUMMARY.

1. The distribution of pepsin between particles of gelatin or coagulated egg albumin and the outside solution has been found to be equal to the distribution of chloride or bromide ion under the same conditions. This is the case from pH 1 to 7, and in the presence of a variety of salts.

2. Pepsin is therefore probably a monovalent anion.

3. Under certain conditions the enzyme may be adsorbed on the surface of the protein particles. This reaction is irreversible and is markedly influenced by the presence of low concentrations of electrolytes.

FURTHER STUDIES ON NEW METHODS IN THE PHYSIOLOGY AND PATHOLOGY OF PLANTS.

By C. B. LIPMAN and A. GORDON.

(*From the Plant Nutrition Laboratory, University of California, Berkeley.*)

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In order to study the effects of solutions on the life processes of a tree it is desirable to introduce them directly into the trunk, since when added to the soil they may be greatly modified and it is often difficult to determine what actually enters the tree. The direct injection of trees and other plants with solutions seemed until recently to offer insuperable difficulties from a practical standpoint. In the last few weeks we have learned of attempts which have constituted improvements on the early methods of tree injection, as it may be called, but none of them seems to have overcome the practical difficulties which present themselves. The method which we have developed, within the last 2 years or more, independently of any knowledge of similar attempts, has already proven eminently successful.

A partial list of investigations of a similar nature which, for some reason or another, have failed of success is given by Miss Rumbold.[1]

Method of Tree Injection.

At a distance of several inches to 2 feet from the ground, a hole is bored into the trunk of the tree, almost at right angles to the direction of the trunk. For this purpose, a wood auger is employed with a bit of $\frac{3}{8}$ to $\frac{1}{2}$ inch in diameter. The hole is bored about three-fourths through the diameter of the trunk of the tree. The shavings are removed from the hole and a glass tube of 2 or 3 inches in length just fitting into the hole is inserted, but only deep enough to make a stable connection. A specially prepared sealing wax is melted around the junction of the glass tube and the tree trunk so as to seal the hole from

[1] Rumbold, C., The injection of chemicals into chestnut trees, *Am. J. Bot.*, 1920, vii, 1.

the outer air. By means of a long rubber tube, the glass tube is connected to the reservoir containing the solution.

The Proper Height for the Reservoir.

In order to determine the most effective height for the reservoir tests were made at the level of the hole in the trunk, 80 inches above the hole, 120 inches above the hole, and 20 feet, above the hole. The data in Table I indicate the time necessary to introduce 3 liters of solution into a pear tree approximately $6\frac{1}{4}$ inches in diameter with a hole $\frac{5}{16}$ of an inch in diameter in the trunk, and about $4\frac{1}{2}$ inches in depth.

The penetration of solutions into the tree, when the reservoir is at the level of the hole in the trunk, is extremely slow. The rate of penetration increases, however, in geometric ratio as the height of the reservoir is increased, until a height of about 7 feet is reached.

TABLE I.

Time Necessary to Inject 3 Liters of Solution into a Pear Tree.

Height of reservoir from point of injection.			
At level of point of injection.	80 in. above point of injection.	120 in. above point of injection.	20 ft. above point of injection.
20 hrs., 11 min.	48 min., 20 sec.	20 min., 20 sec.	12 min., 30 sec.

After that the rate still continues to increase, but not in the same proportion. Nevertheless, with the reservoir 20 feet above the hole at which the injection is made, or approximately the height of the tree, the rate of penetration of the solution is greatest and 3 liters of solution are absorbed by the tree in 12 to 13 minutes. Raising the reservoir above the height of the tree does not increase the rate of flow. In fact, raising the reservoir considerably above the height of the tree decreases the rate of flow of the solution.

It is worthy of note that the rate of flow is not the same under all conditions. The rates given in Table I represent periods of fairly dry weather with the soil below optimum in moisture content and with full foliage on the tree. While we have shown that solutions will also flow from our reservoirs into a leafless tree, and when the soil is very wet, the rate is five or six times slower under those conditions than it is when the foliage is on the tree.

Amounts of Water Which Trees Absorb.

We next proceeded to determine how much water or solution can be injected into one tree, noting the rate of absorption of every successive 3 liters of solution. The data given in Table II indicate the results obtained in this experiment.

TABLE II.

Time Required for Injection of Successive Doses of 3 Liters of Water into the Tree. Each dose 3 liters.

Dose No.								
1	2	3	4	5	6	7	8	9
11 min.	12 min.	12 min.	12 min., 30 sec.	11 min., 30 sec.	12 min., 30 sec.	13 min.,	13 min., 30 sec.	15 min.

Dose No.								
10	11	12	13	14	15	16	17	18
17 min.	19 min.	24 min., 30 sec.	26 min., 30 sec.	31 min., 30 sec.	33 min.	37 min.	36 min.	46 min., 30 sec.

Dose No.		
19	20	21
1 hr., 1 min.	1 hr., 43 min., 30 sec.	2 hrs., 37 min., 30 sec.

Total injection into one tree approximately 63 liters in about 12 hours.

It is clear from the data submitted that pear trees are capable of absorbing large quantities of solution. In this case, about 63 liters of solutions were injected into a pear tree in about 12 hours. The rate of absorption does not diminish very markedly, moreover, until approximately 30 liters have been absorbed. After that there is a steady increase in the time required for the absorption of 3 liters of solution. Beyond the eighteenth injection of 3 liters, every successive increment of 3 liters required a much greater period for its absorption by the tree. Here again, it is to be noted that the data may be relative and not absolute when a changing environment is brought into play.

The Distribution of the Solution in the Tree after Injection.

We have obtained a variety of evidence that solutions injected into trees become quickly distributed throughout all portions thereof. We may now discuss some of this evidence.

If two holes are bored in the tree trunk several inches or more apart and the injection apparatus is attached to the lower hole, it is found that in a short time, there is a discharge of solution from the upper hole. If, on the other hand, the injection apparatus is attached to the upper hole, solutions will likewise issue from the lower hole. Further, if a branch is broken off in the upper part of the tree during an injection of solution, at the point of its attachment to another branch, the solution issues from the broken surface of the main branch which remains attached to the tree.

Our observations were further confirmed as regards rapid distribution of injected solutions through trees by the following experiments on the injections of salts. 1 gm. of $NaHAsO_4$ was dissolved in 3 liters of distilled water and the solution injected into a 10 year old pear tree. 24 hours later, branches and leaves from different parts of the tree were tested for As by the Gutzeit method. Positive tests for As were obtained in all cases. In 48 hours, moreover, all the leaves on the tree turned black and presented a burned appearance.

Again, pear trees and an apricot tree each received injections of 2 or 3 gm. of KI. Tests of leaves and branches showed positive tests for I_2 in all cases.

To observe the path of the circulation of the solutions in the experimental trees, 6 liters of concentrated solution of methylene blue in one case, and 3 liters of solution of methyl green in another case, were injected. Two other trees received, respectively, 3 and 6 liters of concentrated eosin solution. Cross-sections of leaf, limb, and trunk tissue were examined under the microscope. The staining with eosin was found to be clearly limited to definite areas corresponding to the xylem and phloem regions. In passing, the different effects of the different dyes may be noted. With methylene blue and methyl green the trees remained uninjured, despite the concentrated solutions used. With eosin, however, the leaves on both trees showed marked injury within 3 days and on the 4th day had fallen to the ground.

Further evidence on the effective distribution of solutes from the injected solutions will be furnished in connection with the discussion on treatment of chlorotic citrus trees.

Some Effects of the Same Salt on Different Kinds of Trees.

One and the same salt, when injected into different varieties of fruit trees may produce different and specific effects, as exemplified by the following experiments:

5 gm. of KI dissolved in 2,500 cc. distilled water were injected into a pear tree. The tree was greatly injured, the leaves and branches looking as if they had been burned. The tree was not killed, however, and in 4 months had recovered. In another pear tree, 1 gm. of KI was injected. The margins of the leaves on the tree had the same burned appearance manifested by those treated with the more concentrated KI solution, but the tree otherwise was not injured and recovered completely.

On the other hand, when 2.7 gm. of KI were injected into an apricot tree, the effect was strikingly different. Within 24 hours, small circular, dark spots appeared all over the leaves. 1 week later, the tissue in these spots dried up and fell out, leaving the leaf with the appearance of a sieve. The unaffected parts of the leaves appeared to be functioning normally, however. As many as 200 holes were found on one leaf under the conditions named. These observations would seem to point to the existence of some irregularly distributed substance in the apricot leaf, which reacts with KI with resulting injury. Evidently, if such a substance exists in the pear leaf, its distribution in the leaf is wholly different from that in the apricot leaf.

The Effects of KCN and Dicyandiamide.

On December 22, 1923, a pear tree received an injection of 3 liters of solution containing 1.2 gm. $NH_2(NH:)C \cdot NH \cdot C:N$. The tree was distinctly injured but recovered in a period of 5 months. Another tree received an injection of only 0.25 gm. $NH_2(NH:)C \cdot NH \cdot C:N$, but remained apparently uninjured. In both cases, however, the leaves remain small to the present, and growth seems retarded.

Injections of KCN were tried in other trees simultaneously with

the dicyandiamide injections. 8 gm. of KCN were injected into a pear tree. The latter was very severely injured, but within 7 months had recovered, and except for a retarded growth, shows no sign of permanent injury. These experiments appear to have far-reaching implications, which will be considered in part below and in part in other papers.

The Tolerance of Trees for High Concentrations of Salts.

The foregoing discussion has shown that while trees may be easily injured by certain chemical compounds, they may recover from injections of very large amounts of certain toxic substances. This seemed to indicate that non-toxic salts, among them those considered essential to plant life, might with impunity be injected into trees in high concentrations. This, indeed, proved to be the case in a series of tests whose results are set forth in Table III. The salts were injected into pear trees in concentrations of 0.3 N. It seems best to discuss separately each type of salts used.

Magnesium Salts.—Contrary to the generally accepted belief among plant physiologists, magnesium salts are shown in Table III to be not only non-toxic, but decidedly stimulating while other salts may act as deterrents to growth. The present indications are that trees treated with Mg salts produce larger leaves, are much deeper green in color, and, for the second season, seem to set more fruit buds of greater vigor than the ordinary.

Calcium Salts.—Calcium salts proved to be more toxic, or at least deterrent to growth, than potassium salts in the amounts here employed and when injected in the early part of the growing season. The leaves are light in color and rather small. The tree gives the impression of a more open growth instead of the dense growth noted for magnesium-treated trees. Both $CaH_2(PO_4)_2$ and $CaH_4(PO_4)_2$ are distinctly deterrent in effect whereas corresponding magnesium phosphates are not toxic and are even stimulating.

Potassium Salts.—Contrary to what would be expected from *a priori* considerations, potassium salts, under the conditions of our experiments, are much more toxic than magnesium salts and in no case stimulating like the magnesium salts. Like the trees receiving calcium salts, the leaves of trees receiving potassium salts have a

TABLE III.

Effect of Different Salts on 10 Year Old Pear Trees.

Injection of one equivalent per tree.

Kind of salt.	Tree No.	Time of injection.	Condition in Apr., 1924.	Condition June to Sept., 1924.	Remarks.
$NaNO_3$	1	7 Dec., 1923.	C	C	
	2	14 Feb., 1924.	"	"	Foliage profuse; pale green. First to become dormant.
	3	15 Mar., "	D	"	" " "
	1	7 Dec., 1923.	C	"	Foliage sparse; poor growth.
KNO_3	2	14 Feb., 1924.	"	"	" " " "
	3	15 Mar., "	"	D	" " " "
	1	7 Dec., 1923.	A	A	Foliage dense; dark green. Fruit heavy. Last to become dormant.
$MgNO_3$	2	14 Feb., 1924.	"	"	" " "
	3	15 Mar., "	"	"	" " "
	1	7 Dec., 1923.	B	B	Good growth. Good set of fruit.
$Ca(NO_3)_2$	2	14 Feb., 1924.	C	C	Fair "
	3	15 Mar., "	E	D	Leaves irregular in size. Poor growth.
	1	22 Dec., 1923.	C	C	
KH_2PO_4	2	14 Feb., 1924.	"	"	Foliage pale green.
K_2HPO_4	3	15 Mar., "	E	D	" " " ; poor growth.
	1	22 Dec., 1923.	A	A	" heavy; dark green.
$MgHPO_4$	2	14 Feb., 1924.	"	"	" " " "
	3	15 Mar., "	"	"	" " " "
$CaHPO_4$	1	7 Dec., 1923.	C	C	Scarcely fair growth.
	1	11 Sept., "	"	"	Heavy bloom. Heavy June drop.
$CaCl_2$	2	8 Mar., 1924.	"	"	
	3	15 " "	D	"	Good foliage. Fair growth.
	1	8 " "	C	"	Dense "
$CaSO_4$	2	15 " "	"	"	Heavy June drop.

A = excellent growth; B = good growth; C = fair growth; D = poor growth; E = decided injury.

light green color, are small, and show a characteristic open growth. In general, potassium nitrate, at the concentrations we employed, retards growth in these experiments more than calcium nitrate or sodium nitrate.

General Remarks on Experiments with Mg, Ca, K, and Na Salts.

It is of importance to realize, in connection with the discussion in the foregoing section, that the effects of the salts in question may prove to be quite different at lower concentrations. There can be little doubt that Ca and K salts in lower concentrations than those considered above, will have stimulating rather than retarding effects. Even if this should prove so, however, it cannot alter the fact that, contrary to all our preconceptions, Mg salts are highly stimulating, even at very high concentrations, and in a variety of forms. Moreover, we have been able to show that the period of the year in which the salt injections are made is a factor of great importance. A certain salt at a given concentration may be a deterrent when injected at one season of the year and a stimulant to growth when injected at another season.

The Treatment of Chlorosis.

It has long been considered reasonably certain that chlorosis in plants is caused by a deficiency in available iron. Many attempts have been made to supply the deficiency by painting cut surfaces with $FeSO_4$ or by spraying. Some of these attempts with grape vines and pineapples have been reported as highly successful, others as questionable. No successful application of the idea as regards chlorotic trees has come to our notice, in spite of the fact that attempts have been numerous. The reason for these failures has been that spraying trees has not resulted in introducing enough $FeSO_4$ into the leaf cells, as the senior author proved in his own experiments on orange trees 10 or more years ago. On the other hand, the total failure of introducing $FeSO_4$ into the soil where chlorotic trees are growing is easily accounted for. Chlorosis usually occurs in soils whose solutions have a high enough hydroxyl ion concentration to precipitate out nearly all the iron salts which may be introduced.

Taking these facts into consideration we determined to attack

the chlorosis problem by means of injections. Since this is only a preliminary account of our experiments, it is unnecessary to give details of this work here. In general, however, we may state that injections of 0.1 to 7 gm. of $FeSO_4$ in lemon trees which have been chlorotic for some years, have caused the foliage to become green and induced renewed growth and the production of green vigorous-looking branches. It is interesting to note in this connection that the $FeSO_4$ gives evidence of its effects first by the turning green of the vascular systems in the veins of the leaves, and then by the gradual spread of the green color to the intervening tissues of the leaf. The general effect is rapid enough to show in a period of a few weeks. The permanence of this effect can only be determined later.

Other Applications of the Injection Method.

Now that we have proved the practicability of the injection method, many avenues for its application present themselves. If, as we have some reason for believing, mottle leaf of citrus trees is caused by a deficiency in calcium[2] under certain conditions, our injection method should give us the key to the solution of that vexed and economically important problem. If trees can withstand the injection of as much material as our experiments indicate we should be able to attack the problem of freeing them from bacteria, fungi, and possibly insects. We hope to have interesting experimental data to report in the near future in regard to these and similar problems.

It should be added that we have found our method of injection to be applicable to small plants, as well as to trees.

[2] Kelley, W. P., and Cummins, A. B., Composition of normal and mottled citrus leaves, *J. Agric. Research*, 1920–21, xx, 161.

AN INTERFACIAL TENSIOMETER FOR UNIVERSAL USE.

By P. LECOMTE DU NOÜY.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 5.

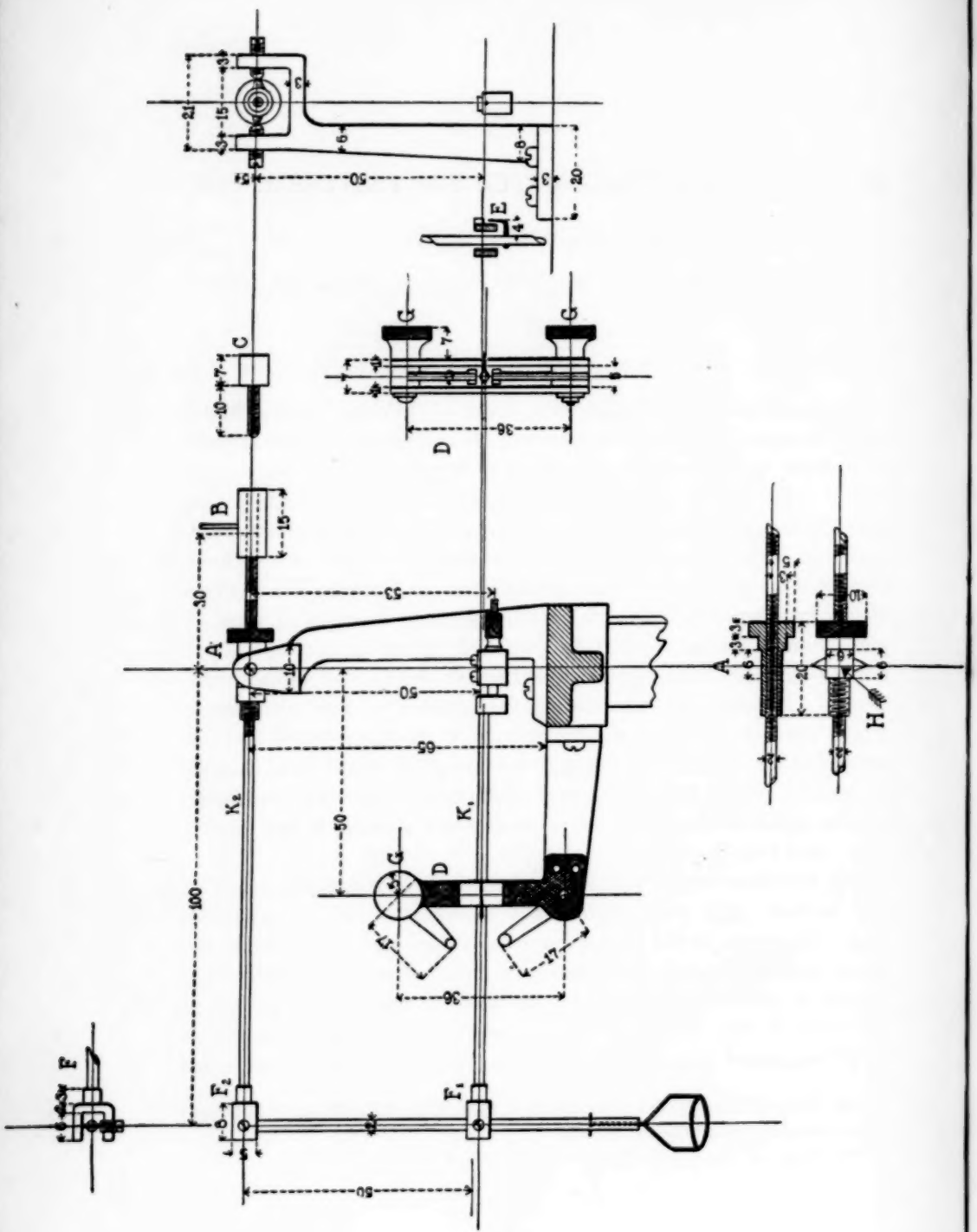
(Accepted for publication, April 6, 1925.)

The important and fundamental works of Quincke, Hardy, McC. Lewis, Morgan, and lately Harkins and his collaborators have thrown much light on the behavior of liquids at interfaces, and it may seem hardly necessary to develop a new method in order to study this phenomenon, as those existing have reached a high degree of accuracy. However, if the values of the interfacial tensions of pure liquids have been well established by these investigators and others, the changes of the interfacial tension due to adsorption in function of time—a phenomenon similar to that first studied in our laboratory[1]—have not, to our knowledge, been studied so far, for lack of proper technique. Consequently, considering the number of new phenomena which were brought to light by the use of the tensiometer first described in this Journal 6 years ago[2] we thought it might be of interest to devise a similar instrument with which measurements of interfacial tension could be made in a few seconds, conveniently, in any laboratory, to 0.1 dyne.

Our first idea was to utilize the tensiometer as it actually exists on the market, with perhaps a slight modification of the platinum ring. However, as the ring can only be pulled upwards, it limits the measurements for water interfaces, to liquids floating on water; *i.e.*, having a specific gravity < 1. In order to render the instrument universal, it was decided to fix the ring to a rigid support, so that it could be pressed down by means of the torsion wire, as well as pulled

[1] du Noüy, P. L., Papers I–X and XII, *J. Exp. Med.*, 1922–25, xxxv–xli; Paper XI, *J. Gen. Physiol.*, 1923–24, vi, 625.

[2] du Noüy, P. L., *J. Gen. Physiol.*, 1918–19, i, 521.


A
B
C
D
E
F
G
H
K1
K2
F1
F2
100
50
30
53
65
36
20
15
10
7
4
17

up. This rigid support is kept in its perpendicular position by means of an articulated parallelogram, $K_1F_1K_2F_2$ as seen in Text-fig. 1. The length of both horizontal arms can be adjusted so as to change the moment of the force applied to the ring, and to make the instrument direct reading.

The balance of the whole moving system is grossly obtained by a counterweight, B, and the finer adjustment of the zero is realized, as in the ordinary tensiometer, by twisting more or less the end of the torsion wire.

The instrument is standardized with weights, in the usual way by considering that the formula

$$\gamma = \frac{M \cdot g}{2L}$$

in which M = mass in gm. (arbitrary) placed on the ring, g = 981, L = mean length of circumference of ring (6 cm., for instance), gives a value γ which must be the figure read on the dial, when the equilibrium is attained. In case the figures do not coincide, the length of both arms, the upper one, K_2, and the lower one, K_1 is slightly increased or shortened, according to the difference. The weights are then removed and the vernier is brought back to zero by means of the knob. If, in this position, the needle E on the lower arm, does not coincide with the index line on the post D, it is set by means of the torsion wire, and the operation must be repeated with weights. It takes usually less than 10 minutes to standardize the instrument perfectly. When this is done, it is, of course, standardized not only for upward pull (vernier on upper part of the scale) but also for downward push (vernier on lower part of the scale) and the readings are identical in both ways. The scale is graduated throughout the circumference of the dial.

A few measurements were taken with substances studied by Harkins, Brown, and Davies (drop method).[3] No attempt was made to secure pure liquids, our only aim being to check the soundness of the method. As Harkins and his collaborators took special pains in order to secure absolutely pure materials the discrepancies found

[3] Harkins, W. D., Brown, F. E., and Davies, E. C. H., *J. Am. Chem. Soc.*, 1917, xxxix, 356.

between their values and the tensiometric values may be accounted for in this way for the substances spreading on water (Table I). However, the values disagree entirely for the liquids which do *not* spread on water, namely those having an interfacial tension greater

TABLE I.

Surface Tension and Interfacial Tension against Water.

Experiment No	Liquid.	Temperature.	Dynes cm. γ	Interfacial tension against water. γ_w	Difference. $\gamma - \gamma_w$	Sp. gr.
		°C.				
1	Chloroform	25.0	27.5	27.9	+0.4	1.526
2	Isoamyl alcohol	23.5	24.2	4.4	−19.8	0.825
3	Octyl alcohol	25.0	28.6	10.8	−17.8	0.837
4	Benzyl alcohol	25.0	43.3	4.8	−38.5	1.023
5	Ethyl iodide	25.0	32.3	36.6	+4.3	1.944
6	Ethyl ether	23.0	18.0	10.9	−7.1	0.718
7	Ethyl phthalate	25.0	40.8	16.4	−24.4	1.126
8	Ethyl carbonate	25.0	29.0	11.9	−18.1	0.978
9	Oleic acid	25.0	34.2	12.8	−21.4	0.708
10	Benzene	25.0	29.0	32.3	+1.3	0.880
11	Toluene	25.0	28.0	32.5	+4.5	0.882
12	*m*-xylene	23.0	30.0	35.0	+5.0	0.878
13	Castor oil	23.0	36.4	15.4	−21.0	0.950
14	Olive oil	23.0	33.0	17.0	−16.0	
15	Dimethyl aniline	26.0	38.4	21.0	−18.9	0.955
16	Diethyl aniline	25.0	35.4	21.3	−14.1	
17	Turpentine	25.0	29.2	13.7	−15.5	
18	"Non-spreading liquids," according to Harkins. Carbon bisulfide	20.0	34.0	33.8	−0.2	1.259
19	Paraffin oil	25.0	32.0	47.0	+15.0	0.850
20	Carbon tetrachloride	23.0	28.0	40.0	+12.0	1.608
21	Bromoform	25.0	39.5	27.5	−12.0	
22	Liquid petrolatum Squibbs	24.0	33.0	49.0	+16.0	

than the difference between the surface tension of water and that of the liquid. In this case, no tension is greater than the sum of the other two, and a triangle of forces is possible and real.

Nevertheless, and without questioning the accuracy of Harkins' values for the interfacial tension between water and this special class

TABLE II.

Comparison between Interfacial Values.

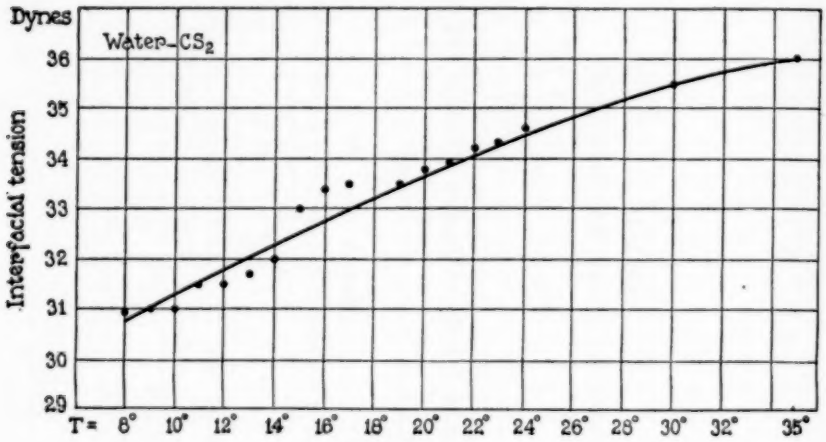
No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	"Non-spreading." 18	19	20	21	22
Tensiometer (du Noüy).	27.9	4.4	10.8	4.8	36.6	10.9	16.4	11.9	12.8	32.3	32.5	35.0	15.4	17.0	21.0	33.8	47.0	37.2	27.5	49.0
Drop method (Harkins).	27.7	4.42	8.52	4.75	40.02	10.7	16.27	12.86	15.5	34.97	36.10	37.6	22.63		25.78	54.09		52.63	40.85	55.32
Cathetometer (Quincke).														18.2		37.4				

TABLE III.

Interfacial Tension as a Function of Temperature.

Temperature, °C.	8	9	10	11	12	13	14	15	16	17	19	20	21	22	23	24	30	35	40	45
Interfacial tension, water-CS_2	30.9	31.0	31.0	31.5	31.5	31.7	32.0	33.0	33.4	33.5	33.5	33.8	33.9	34.2	34.3	34.6	35.5	36.0		
" " water-ethyl ether.	9.5	9.5			9.7		10.0	10.3		10.4	10.6		10.9			11.2	11.7	11.9	12.2	12.9

of substances, it might be pointed out that Quincke,[4] using a cathetometric method, gave, for the interfacial tension water-CS_2, the value 42.6 dynes. As he finds 82.5 for the surface tension of water, while we find 72.8, one might think that it is permissible to reduce this value 42.6 in the ratio $\frac{82.5}{72.8}$ and one obtains 37.4. Our value for water-CS_2 is 33.8, and that of Harkins, 54.09. Quincke's method seems quite reliable, and his figures are in good agreement with Harkins' and ours for water-chloroform (26.5 corrected as above) and water-olive oil (18.2). The difference between the values of Harkins' and those of Quincke and ourselves is probably due to the fact that Harkins



TEXT-FIG. 2.

measures the dynamic tension, while we measure the static value. Since Professor Harkins states that his "method is undoubtedly the most accurate of all those which have been devised, but [that] it has given the most inaccurate results because the corrections which have been used by workers other than ourselves are incorrect," we feel quite certain that his values are standard, and we do not pretend to compete with such a definite statement. Our aim was to devise a method which, although far from claiming to be the most accurate, would nevertheless not require corrections so important as to render it inaccurate in the hands of others. In other words, a simple, rapid,

[4] Quincke, M. G., *Phil. Mag.*, 1871, xli, series 4, 245.

convenient technique which would make it possible to study the adsorption at interfaces and give the static value of interfacial tension, just as the tensiometer gives the static value of surface tension. The action of temperature will be very easy to investigate. From a first series of experiments with water-CS_2, we have gathered evidence that, contrary to what happens at the air-liquid surface the interfacial tension increases with increasing temperature. The same holds true for ethyl ether and octyl alcohol. Harkins observed the same phenomenon for octyl alcohol also and for heptaldehyde and heptin, but did not report the temperature coefficient of ethyl ether nor that of carbon disulfide.[5] See Table III and Text-fig. 2.

These measurements were repeated four times with two different sized rings (circumference, No. 1 = 6 cm.; No. 2 = 12 cm.) in order to increase the sensitivity. In the four cases the values checked within a few tenths of a dyne, the four cases showing the same bump in the curve between 14 and 19 degrees. Around 35°C. the measurements became very difficult owing to the bubbles forming permanently at the interface.

[5] Harkins, W. D., and Cheng, Y. C., *J. Am. Chem. Soc.*, 1921, xliii, 35.

EXPLANATION OF PLATE 5.

FIG. 1. Interfacial tensiometer, with 6 cm. ring.

FIG. 2. Appearance of the interface between water (top) and carbon tetrachloride (below) when the ring is pressed down. Picture taken just before breaking, in the state of equilibrium.

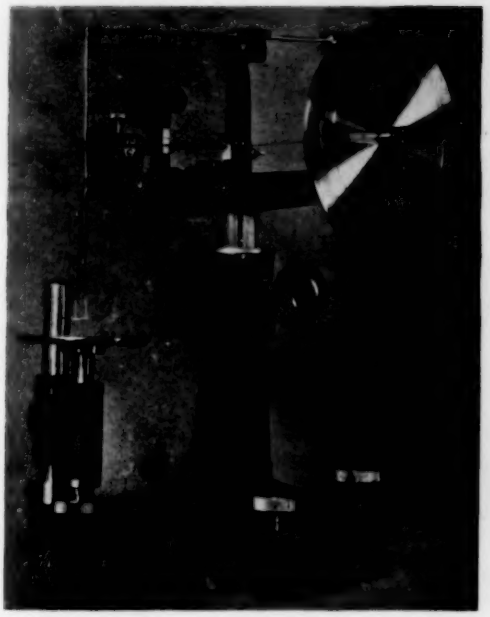


FIG. 1.

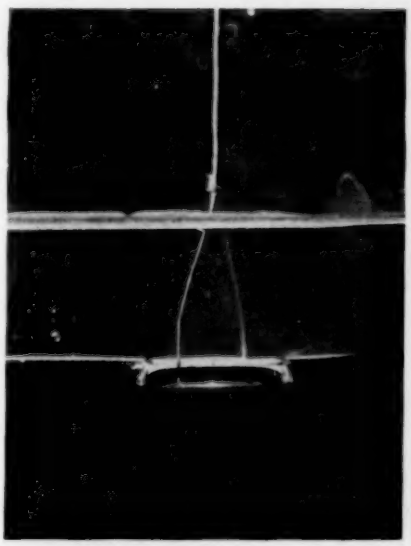


FIG. 2.

(du Noüy: Interfacial tensiometer.)

CONTRASTS IN THE CELL SAP OF VALONIAS AND THE PROBLEM OF FLOTATION.*

By W. J. V. OSTERHOUT and M. J. DORCAS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge, and The Rockefeller Institute for Medical Research.)

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It has been shown[1] that the marine alga *Valonia macrophysa*, Kütz. (collected at Bermuda), has the power to absorb K and to store it up so that its concentration becomes much greater in the cell sap than in the sea water. This is also the case with *Valonia utricularis* (collected at Naples) according to Meyer and Hansen.[2] A similar behavior might be expected in other Valonias but the authors find that this is apparently not the case with the large species found at Bermuda which is commonly referred to *Valonia ventricosa*, J. G. Agardh.[3]

Table I gives a comparison of the analyses of Bermuda sea water, and of the saps of *V. macrophysa* and *V. ventricosa*.

It will be noted that the proportion of K to Na in *V. macrophysa* is about 5.72:1 while in *V. ventricosa* it is about 0.0278:1.

* Contributions from The Bermuda Biological Station for Research, No. 149.

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225. There is some doubt regarding the identity of this species.

[2] Meyer, A., *Ber. bot. Ges.*, 1891, ix, 77. Hansen, A., *Mitt. zool. Station Neapel*, 1893, xi, 254. Czapek (Czapek, F., Biochemie der Pflanzen, Jena, 2. Auflage, 1920, ii, 353) mentions (without quoting the source) an analysis of *V. utricularis* in which Na predominates over K but the analysis was presumably made on whole plants and there may have been a good deal of contamination by sea water, as is suggested by the high values for SO_4.

[3] *Cf.* Collins, F. S., and Hervey, A. B., The algæ of Bermuda, *Proc. Am. Acad. Arts and Sc.*, 1917, liii, 51. Britton, N. L., Flora of Bermuda, New York, 1918. Algæ by M. A. Howe. *V. ventricosa* is cast up on beaches as clear green, balloon-shaped cells sometimes as large as pigeon eggs and is known locally as "sea bottles." In the sun the cells soon bleach and become colorless.

In the latter the concentrations of Na and K are nearly the same as in sea water.

The table shows that Mg, which is absent from *V. macrophysa*, occurs in *V. ventricosa*, though in lower concentration than in sea water; Ca is present in both cases but in much lower concentration than in sea water.

In both cases NO_3 is present although the sea water gives no indication of it by the ring test. In both species the halide content, and osmotic pressure are higher than in sea water.[4] The pH of the sap is about the same in both, approximately 5.9, while that of the sea water is about 8.2.

There is practically no Br, PO_4, or SO_4 in the sap[5] of intact cells of either species, but SO_4 begins to diffuse in as soon as injury occurs and this serves as a useful indicator of normal condition.

In order to gain an approximate idea of the amount of organic matter the loss on ignition was determined. The procedure employed in the case of *V. macrophysa*[1] was followed in the main except that the filtration was omitted on the ground that some Ca and Mg might be retained on the filter as carbonate. The sap was dried to constant weight in an oven at 105°C. and ignited just below red heat. Water was added (to replace any water held between the crystals which may have been driven off by ignition) and the residue was again dried to constant weight at 105°C. The difference in weight before and after ignition amounted to 2.09 parts per thousand: the corresponding figure obtained by Van der Pyl for *V. macrophysa* is 1.433 parts per thousand.

These striking differences between two species of *Valonia* raise the question whether it is justifiable to assume, as is often done, that close relationship involves similarity in chemical composition and

[4] Determinations of conductivity were made by Mr. L. R. Blinks who found that the conductivity of the sap of *V. ventricosa* to be about the same as that of sea water. In *V. macrophysa*, however, the conductivity is about 20 per cent higher than that of sea water, as is to be expected in view of the larger proportion of KCl, which has a higher specific conductivity than NaCl.

[5] Freshly collected normal cells show only a trace of turbidity when $BaCl_2$ is added to the acidified sap: this very slight turbidity may be due to the precipitation of organic matter.

metabolic processes. The implications of this question are far reaching. If it is possible for nearly allied forms to differ so profoundly it is evident that we have as yet no satisfactory conception of the nature of the variables involved in differentiation or in the development of specific characters.

These differences suggest another question of considerable im-

TABLE 1.*

Molecular Composition Expressed as Per Cent of Halide (Cl + Br).

	Bermuda sea water.	Sap of *Valonia macrophysa.*	Sap of *Valonia ventricosa.*
Cl + Br†	100.00	100.00	100.00
Na	85.87	15.08	92.80
K	2.15	86.24	2.58
Ca	2.05	0.288	1.36
Mg	9.74	Trace ?	2.49
SO_4	6.26	Trace ?	Trace ?

* The figures for sea water and for the sap of *V. macrophysa* are based upon the results of L. M. Van der Pyl (*cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225). The methods described in that paper were followed in the work on *V. ventricosa* except that the halide was determined volumetrically and the Ca, after separation as oxalate, was determined by titrating against $KMnO_4$, instead of gravimetrically.

In the table the sea water appears to have an excess of anions over cations of 0.825 per cent (each bivalent ion being counted as two univalent ions): this is due in part to the fact that the halide was determined gravimetrically and called Cl, no account being taken of the Br present, which in molar terms is approximately 0.14 per cent of the Cl. This procedure would make the Cl appear too great (owing to the greater atomic weight of Br). On the other hand no account was taken of carbonates.

In the sap of *V. macrophysa* the table shows an excess of cations amounting to 1.9 per cent; in the case of *V. ventricosa* the apparent excess of cations is 3.08 per cent. This is undoubtedly due in part to the fact that no account was taken of carbonates, nitrates, or organic anions.

The extraction and analysis of the sap of *V. ventricosa* were carried out by the junior author.

† Br is present in sea water but absent from the sap of both species of *Valonia.*

portance, whether Na or K predominates in the sap[6] of plant and animal cells. For the study of this question, it is desirable to employ cells whose sap can be obtained without alteration, as in the case of *Valonia*. The fresh water plant *Nitella* fulfills this condition. Analysis of the sap of *Nitella*[7] from Woods Hole (*Nitella* "A") shows approximately equal amounts of Na and K: in a species collected near Cambridge (*Nitella* "C") there is a little more K than Na. Hoagland and Davis[8] state that in *Nitella clavata*, taken from pond water, the proportion of K to Na in the sap is as 5.43 to 1. In the case of large cells in tap water, however, they report that Na and K were more nearly equal and in small cells in culture solution Na predominated over K.

In these cases the sap could be extracted without contamination or alteration. Where this is not possible we cannot be sure of its composition. Ordinary ash analyses, made without extracting sap, do not tell us whether substances are present in soluble or insoluble form, whether they are located within the cell or in intercellular spaces. But such analyses may nevertheless give us some idea of the general situation.

Ash analyses show a decided predominance of K over Na throughout the flowering plants and mosses.[9] In the case of marine algæ[10] they often indicate a preponderance of Na over K, but this might be due to sea water held in the cell walls or adhering to the surfaces of the plant.

The analyses of animals are less consistent than those of flowering

[6] The word sap is here used to designate the fluid within the cell whether imbibed in the protoplasm without formation of vacuoles, as is commonly the case in animals, or forming vacuoles, as in plants.

[7] The cells were carefully washed, rinsed with distilled water, and quickly crushed. The crushed mass was subjected to pressure and the liquid obtained by this procedure was filtered. In this case there is opportunity for alteration of the sap but Hoagland and Davis[8] found that sap extracted in this way did not differ in respect to Na and K from sap collected by squeezing it out from individual cells in such a manner as to prevent alteration.

These two species are called "A" and "C" pending identification.

[8] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

[9] Czapek, F., Biochemie der Pflanzen, 2. Auflage, Jena, 1920, ii, 369 ff.

[10] *Cf.* Czapek, F., Biochemie der Pflanzen, 2. Auflage, Jena, 1920, ii, 353.

plants but K seems to predominate over Na in the majority of cases. Erythrocytes are of special interest. In some cases they contain little or no Na and there is reason to believe[11] that the large amount of K which is present exists in solution as an inorganic salt. On the other hand there are species whose erythrocytes contain mostly Na with very little K.

It is therefore apparent that we are not warranted in concluding that K invariably predominates over Na in the sap of living cells.

Another interesting problem is suggested by the fact that the dissimilarity in composition of the two Valonias brings about a marked difference in their behavior in that *V. macrophysa* sinks in sea water while *V. ventricosa* floats.

The specific gravities at 25°C. are as follows: sea water 1.0277, sap of *V. macrophysa* 1.0290, sap of *V. ventricosa* 1.0250 (distilled water being taken as 1.0000). In order to see whether the salt composition as ascertained by analysis could account for these differences artificial sea water and saps were made up according to the analyses given in Table I and their concentrations were adjusted until the halide content of the artificial sea water was 0.59 M and that of the saps 0.626 M, which represents a typical condition in respect to sea water and the two saps. The specific gravities are as follows: artificial sea water 1.0285, artificial sap of *V. macrophysa* 1.0285, artificial sap of *V. ventricosa* 1.0252. These figures are in fair agreement with those given above.

Since the cell walls and the protoplasm of both species sink in sea water it is evident that living cells of *V. macrophysa* must sink in sea water as their sap is heavier. The sap of *V. ventricosa* is so much lighter that the cells float despite the weight of the cell wall and protoplasm.

To test roughly whether the differences mentioned above would suffice to account for the behavior of the cells a solution was prepared containing NaCl and KCl in the same molecular proportions and concentration as the sap of *Valonia ventricosa*. Dead cells of *Valonia macrophysa*, placed in this and left until it had diffused in, were then transferred to sea water where they floated for a few minutes, until

[11] *Cf.* Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

the specific gravities of the internal and external solutions became so nearly equal by diffusion that the weight of the cellulose wall and protoplasm caused the cells to sink.

We may ask whether other cells are able to float by means of the mechanism found in *Valonia ventricosa*. Aside from cells which float because of the presence of gas vacuoles or of oil, there are cases like that of *Noctiluca*. From the work of Mrs. Harvey[12] and of Lund and Logan[13] we may conclude that the numerous large vacuoles of *Noctiluca* contain an aqueous solution whose specific gravity is less than that of sea water. Lund and Logan observed that after treatment with an electric current the vacuoles coalesce at the surface and there seems to be a destruction of the plasma membrane at the points where this coalescence occurs; in consequence the acid[14] contained in the vacuoles diffuses out of the cell. After this the cell is no longer able to float.

It is evident that the destruction of the plasma membrane, together with accompanying changes, might allow an outward diffusion of substances (whether contained in the vacuoles or not) and at the same time substances might diffuse in from the sea water. The differences in the specific gravity between the cell sap (whether collected in vacuoles or imbibed in the protoplasm) and the sea water would thus disappear and the cell would sink.

If the cell sap had a composition similar to that of *Valonia ventricosa* this might explain the observation of Mrs. Harvey that when *Noctiluca* is placed in sufficiently dilute sea water it sinks, but after a time rises to the surface. If we assume that *Noctiluca* floats in the same manner as *Valonia* it would sink in dilute sea water[15] and then take up water until its normal osmotic balance was restored (with an

[12] Harvey, E. B., *Papers from the Department of Marine Biology of the Carnegie Institution of Washington*, 1917, xi, 235. (*Carnegie Institution of Washington, Pub. No. 251.*)

[13] Lund, E. J., and Logan, G. A., *J. Gen. Physiol.*, 1924–25, vii, 461.

[14] The sap in the vacuoles of *Noctiluca*, like that of *V. ventricosa*, is much more acid than sea water. *Cf.* Harvey,[12] Lund and Logan,[13] and Lyon, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 284.

[15] The degree of dilution necessary to cause the cell to sink would depend not only on the cell sap but on other constituents of the cell, including those lighter than sea water (such as oil, etc.).

osmotic pressure equal to or greater than that of the dilute sea water): it would then float because of the smaller specific gravity of its cell sap.

If the dilution is so great as to injure the cell and increase its permeability it sinks, as is to be expected, since, for the reason given above, dead cells of *Noctiluca* and *Valonia ventricosa* are unable to float.

The question may now be raised whether the cells of *Valonia ventricosa*, as described above, are to be regarded as normal. It might be suggested that while they are growing their sap resembles that of *Valonia macrophysa*, but that as they begin to die their permeability changes, allowing KCl to diffuse out and NaCl to diffuse in, without, however, admitting SO_4 until a later state in the death process. As a result they would float until they were so nearly dead as to admit SO_4 and would then sink. As a matter of fact when cells of *Valonia ventricosa* which seem perfectly normal are brought into the laboratory they may remain so for a long time but as soon as marked signs of injury appear (*e.g.* disturbance of the chloroplasts) they sink. Sinking, therefore, appears to be a sure sign of death and it is accompanied by the entrance of SO_4.

It should be remembered that these cells not only appear normal but that they differ from dead cells in the content of NO_3, Mg, Ca, Cl, H ion, and organic matter in the sap, and in their osmotic pressure.

The writers have made every effort to obtain young, growing cells. Many of the cells found floating are very small, but this does not constitute proof of normal condition. During the last 2 years a few cells apparently of this species have been found attached. These were very small and floated when detached; they appeared normal in all respects. Unfortunately there seems to be no prospect of obtaining such cells in sufficient quantity for analysis. It therefore seems desirable to publish the facts already obtained, since they suggest important problems, some of which may be attacked by investigation of other material.

SUMMARY.

The marine alga *Valonia macrophysa* contains in its cell sap K and Na in the proportion of 5.72 to 1. In a form regarded as closely

related, *Valonia ventricosa*, growing in similar environment, the ratio of K to Na is as 0.0278 to 1. The former contains no Mg but in the latter it is present. There are other differences in the composition of the sap.

These differences bring about a remarkable contrast in behavior in that *Valonia ventricosa* floats in sea water while *Valonia macrophysa* sinks.

THE PRODUCTION OF CARBON DIOXIDE BY NERVE.

By G. H. PARKER.

(From the Zoological Laboratory, Harvard University, Cambridge.)

(Accepted for publication, April 2, 1925.)

I. INTRODUCTION.

The older physiologists, such as Johannes Müller, believed that nerve impulses were transmitted at a rate comparable to that of light, a belief that led to the interpretation of such impulses as essentially physical phenomena. But when Helmholtz in 1850 measured for the first time the rate of transmission of the impulse on frog nerve and found it to be about 29 meters per second, grounds were given for a chemical interpretation of this action and it was afterwards often compared to the burning of a chain of gun-powder. From this standpoint it is natural to expect that nerve activity would be associated with the absorption of oxygen, the generation of heat, exhaustion, and the production of waste products such as the metabolites water and CO_2. Considerable experimental evidence has been amassed in the last few years to show that oxygen is necessary for the continued activity of nerve, but thus far heat has not been found associated with nerve action. Signs of exhaustion have been claimed only on direct evidence, and of possible metabolites, CO_2, the only one readily identifiable, has been declared present by some investigators and absent by others.

The production of CO_2 by nerve was first specifically claimed by Waller (1896, 1897 a, 1897 b, 1910) about three decades ago when he showed that the electrical properties of a nerve changed on immersing it in an atmosphere rich in CO_2 and that a similar change occurred when the nerve in ordinary air was stimulated. He argued from these observations that the stimulation of the nerve produced within it a metabolite which acted on the fibers in the same way as CO_2 did and that this metabolite was very probably CO_2 itself. Waller, however, was never able to demonstrate by any direct means the presence of CO_2 in the stimulated nerve.

The positive identification of this substance as a product of nerve activity was first accomplished by Tashiro (1913 a, 1913 b, 1917) who devised for this purpose a special apparatus which he called a biometer. In a closed glass chamber a nerve in an atmosphere free from CO_2 was suspended above a drop of aqueous barium hydroxide. If such a nerve should give out CO_2, particles of barium carbonate would be expected to form on the surface of the drop and on carrying out this test such proved to be the case. Tashiro believed that the production of these particles was so regular that the method admitted of quantitative results and he devised a somewhat complicated procedure to attain this end. According to his observations 10 mg. of nerve from the spider crab produced 6.7×10^{-7} gms. of CO_2 in 10 minutes. On stimulation this amount was said to increase to 16×10^{-7} grams or nearly threefold that from the quiescent nerve.

Moore (1917, 1918) immersed a piece of living nerve in a solution of phenolsulphonephthalein and demonstrated that the solution became progressively more and more acid the longer the nerve remained in it. The material producing this acidity could be removed by washing the solution freely with pure air, a result which led him to conclude that the acidity was due to CO_2 and not, for instance, to lactic acid. Moore (1919 a, 1919 b, 1921) was not able to detect any increase of CO_2 on stimulation. In this respect his results were in strong contrast with those of Tashiro.

Tashiro's conclusions were sharply criticised by Bayliss (1915, p. 379) who pointed out that a nerve contained not only nervous tissue but also a considerable amount of connective tissue whose respiratory activity might afford the CO_2 identified by Tashiro. Bayliss also called attention to the fact that since the stimulating electrodes used by Tashiro were within the respiratory chamber, the increase of CO_2 on stimulation might result from the electrical heating of the nerve rather than from increased metabolism.

In consequence of the lack of harmony in the results of Tashiro and of Moore and of the criticism of the whole situation by Bayliss I was led to undertake a reinvestigation of the subject. My work was carried out at the Marine Biological Laboratory at Woods Hole, Massachusetts, and I wish to express to the director, Dr. F. R. Lillie, and to his associates my keen appreciation of the very generous opportunities accorded me at their institution.

II. MATERIAL AND METHODS.

The nerve whose production of CO_2 I proposed to test was the lateral-line nerve of the smooth dogfish, *Mustelus canis* (Mitchell). This nerve is one of the few pure nerves in the vertebrate body. It is composed of very uniform, medullated, sensory fibers. It can be quickly and easily dissected from the side of the fish and yields a very clean smooth trunk, thick anteriorly and diminishing posteriorly as it gives off branches to the lateral-line organs. It is easy to obtain stretches of this nerve 10 centimeters in length.

My first attempts at the determination of CO_2 from this nerve were made by methods that involved the conversion of barium hydroxide into carbonate. But neither the procedure devised by Tashiro (1913 a, 1913 b, 1914) nor that used by Lund (1919) yielded satisfactory quantitative results and my inability to carry out an accurate and close calibration of any apparatus led me to abandon these means. My experience with Tashiro's method agreed closely with that of Adam (1921, p. 362) who found it extremely sensitive as a qualitative test for CO_2 but was unable to use it for reliable quantitative work.

As it was necessary for me to obtain accurate quantitative measurements, I turned, as Bodine (1921 a, 1921 b, 1922) had done, from the barium hydroxide methods to indicator methods. One of these had already been well developed by Osterhout (1918) and modified in a convenient way for my purpose by Jacobs (1920). Moreover, I had already had some experience with this method (Parker, 1922 b) and I therefore attempted to apply it to nerve.

The apparatus that I devised (Fig. 1) consisted of a small glass respiratory chamber and two standard tubes mounted on an inclined stage that could be moved up and down by an electric motor. The respiratory chamber used was of either an open or a closed type. The open type is shown in side view in Fig. 2 and is composed of a glass tube (T) about 15 centimeters long with a tightly fitting ground-glass stopper (S). Following the suggestion of Irwin (1920) this apparatus was made entirely of glass which eliminated leakage due to rubber connections. The glass used was pyrex thus preventing the contamination of the contained fluids by dissolved glass products. The lower part of the tube (I) had a uniform diameter of about

15 millimeters and the upper part (A) one of about 28 millimeters. The two parts of the tube were attached to each other quite out of center but so that the outer face of the narrower part was in one region exactly in line with the outer face of the wider part. The

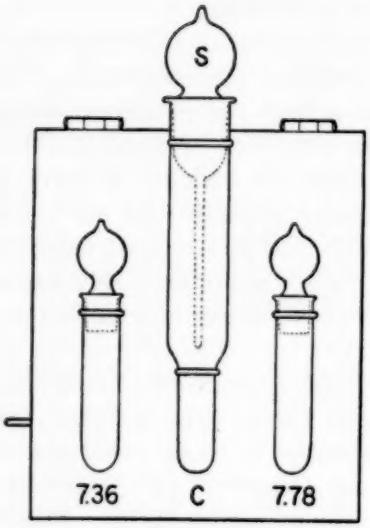

FIG. 1.

FIG. 2.

FIG. 1. Hinged movable stage carrying a respiratory chamber (C), closed type, and two standard tubes containing solutions of phenolsulphonephthalein made up for pH values 7.78 and 7.36.

FIG. 2. Side view of a respiratory chamber (pyrex glass), open type, × ½, composed of a respiratory tube (T) made up of a narrow part (I) filled with the solution of indicator and a wide part (A) containing air and the nerve. The hollow stopper (S) carries on its inner face a rod (R) on which the piece of nerve (dotted line) is laid. Near the base of the rod the stopper is perforated by a hole (H) through which the end of the nerve may be passed for stimulation by electrodes (E) placed in the cavity of the stopper. After the nerve is in position the hole is closed with a stiff mixture of kaolin and vaseline. The closed type of respiratory chamber is like that just described except that it lacks the hole (H) and the electrodes (E).

stopper carried attached to its inner face a bent glass rod (R) which was so shaped that its principal length was parallel with the wall of the wider part of the tube but to one side of its center. The length of this rod was approximately 5 centimeters and when the stopper was in place, the rod extended through almost the whole length of the wider part of the tube but without coming in contact anywhere with its walls. The cavity of the stopper (S) was freely open to the exterior and the thin wall between this cavity and that of the respiratory chamber was perforated by a hole (H). The closed type of respiratory chamber (Fig. 1) was exactly like the open type except that its stopper (S) was not open or pierced by a hole.

In using the closed type of apparatus the narrower part of the chamber was filled with a solution of the indicator, phenolsulphonephthalein, through which air devoid of CO_2 was freely bubbled thus filling the wider part of the chamber with pure air. The piece of nerve to be tested was then laid on the glass rod to which it adhered through its own stickiness and the stopper with its rod and attached nerve was then quickly inserted into the tube before the pure air in the tube could be contaminated to any great extent by the surrounding atmosphere. By this procedure it was possible to close the tube so that it contained indicator, live nerve, and air with only a minimum of CO_2 accidently included at the moment of closure. By steps to be described presently the effect of this small variable amount of CO_2 was eliminated from the final results.

To facilitate the exposure of the indicator to gases that might be given out by the nerve, the respiratory chamber was attached to the movable stage so that the side of the chamber in which the nerve was lodged was uppermost and the stage was set in motion (Fig. 3). This consisted in shifting the stage in such a way that the respiratory chamber was alternately on an incline with its closed end down and on the horizontal. By this means the indicator solution was flushed back and forth six to eight times a minute under the nerve but without coming in contact with it. Thus if any substance passed from nerve to indicator, it did so through the air contained in the respiratory chamber. In this respect the method was believed to be an improvement over that of Moore (1917) in which the nerve was immersed in the indicator solution itself. In the method used in the present

research it was not necessary to remove by aeration the material that occasioned the acidity and thus prove that it was CO_2 as Moore did, for with the nerve separated always from the indicator by air the only substance at all likely to pass across to the indicator and

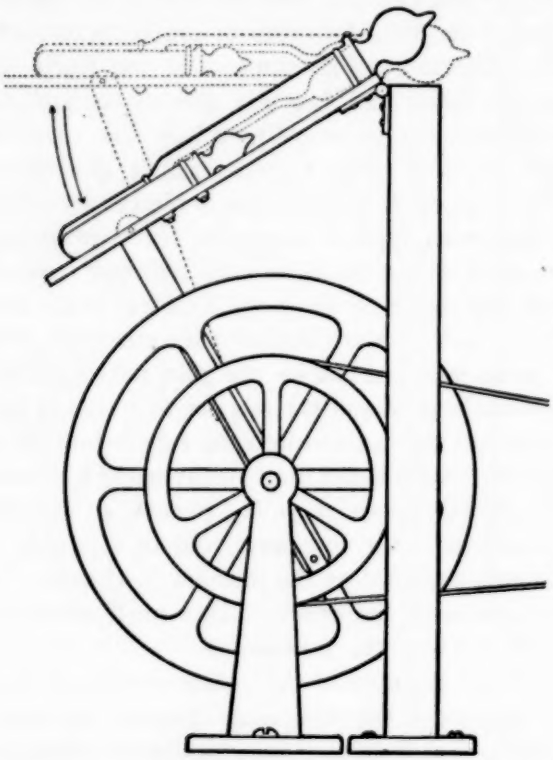

FIG. 3. Side view of the movable hinged stage with its support and a portion of the set of gears by which it was moved up and down. The gears were operated by an electric motor. The extreme positions of the platform are represented in the drawing, the lowest position in full lines, the highest in dotted lines. The gears were so arranged that the stage was lifted and lowered six to eight times a minute.

increase its acidity is CO_2. On grounds that will be presented in a subsequent paper, I believe it can be shown that, contrary to Tashiro's conclusion (1922), ammonia is not given out by a nerve at this stage in its preparation. Hence it is not necessary in experiments such

as are recorded in this paper to take precautions, as suggested by Tashiro (1921), for the removal of this gas.

In the apparatus employed in these tests the washing back and forth of the indicator solution below the nerve had the further advantage of keeping the air in the respiratory chamber well charged with water vapor and thus preventing the nerve from drying, an important precaution in the whole operation.

On testing the nerve in the way described it was found that the indicator gradually changed color in a direction that showed increased acidity. To measure the amount of this change the two standard tubes attached to the stage were used (Fig. 1). Each of these tubes was cut from the same piece of pyrex tubing from which the narrow part of the respiratory chamber had been taken. They were as nearly as possible of the same caliber and thickness of wall as the material in the respiratory tube itself. They were fitted with ground stoppers and filled with standard solutions of the indicator of known pH value (see Osterhout and Haas, 1918, p. 422). The tube on the right contained a solution composed of 8 cc. of 0.2 mol. boracic acid solution, 2 cc. of 0.05 mol. borax solution, and two drops of a saturated aqueous solution of phenolsulphonephthalein. Its color represented a pH value of 7.78. The tube on the left was filled with a solution composed of 9 cc. of 0.2 mol. boracic acid, 1 cc. of 0.05 mol. borax, and two drops of phenolsulphonephthalein solution and represented pH 7.36. The indicator in the respiratory chamber, for reasons that will be given in connection with calibration, was composed of a mixture of 10 cc. of 0.0001 mol. sodium hydrogen carbonate and two drops of a saturated aqueous solution of phenolsulphonephthalein. The solutions in the standard tubes showed a slight deterioration in the course of several weeks; consequently they were set up anew from week to week. The stage on which the three tubes were mounted was covered with white cardboard as a background against which to compare the colors of the several solutions and all such comparisons were made under the illumination of a "daylight" lamp to avoid the uncertainty due to the fluctuating light of the sky.

In beginning a test the respiratory chamber which was in position on the stage was prepared by bubbling through its indicator solution air freed from CO_2 by having been passed over sodium hydroxide.

This air was stored under slight pressure in an appropriate glass reservoir. By this means the small amount of CO_2 contained in the indicator solution was washed out and its color in consequence shifted to a purplish tint well beyond that characteristic for pH 7.78. This operation also incidentally filled the respiratory chamber with air practically free from CO_2. When this point had been reached the pyrex washing tube was withdrawn from the respiratory chamber, the chamber was at once closed by inserting the glass stopper with its rod and attached nerve, and the flushing movements were started. In closing the chamber, as already stated, a small amount of outside air with its contained CO_2 was probably always introduced, but this amount was invariably so slight, as judged by the change in the tint of the indicator, that the whole system still remained well on the alkaline side of pH 7.78. This source of error, therefore, took care of itself, for after the slight readjustment due to this small influx of CO_2 the change in the color of the indicator proceeded in an acid direction with great slowness and regularity. As the tint of the indicator in the respiratory chamber gradually approached that in the more alkaline (pH 7.78) of the standard tubes, it was carefully observed and at coincidence the time was read to seconds by a watch and recorded. The apparatus was kept running continuously and when the color in the respiratory tube agreed with that in the second standard tube (pH 7.36), the time was again read and recorded. The difference between these two times represents the period required by the nerve to produce enough CO_2 to shift the hydrogen ion concentration of the respiratory chamber from 7.78 to 7.36. In ordinary experiments a piece of nerve that was large enough to accomplish this change in from ten to twenty minutes was found most convenient.

In testing the apparatus one of the first trials that was given it was to run it without nervous tissue. Under such circumstances the shift from pH 7.78 to pH 7.36 was accomplished in from two to three days. These trials were made with a thoroughly sterilized outfit and in a laboratory room freely open to the outer air, but the observer was of course in the room and his breath must have continually contaminated the atmosphere. The very slow change shown by the indicator is believed to have been due to the gradual entrance of minute amounts of atmospheric CO_2 into the respiratory chamber by way of

the narrow space between the glass stopper and the neck of the respiratory tube. This leakage, however, required such a long time (two to three days) as compared with that necessary for an observation (ten to twenty minutes) that no attempt has been made to correct for it.

The closed type of respiratory chamber was used for the study of quiescent, unstimulated nerve. To test stimulated nerve the open type of chamber was used. This type (Fig. 2), as already stated, had a stopper whose external cavity opened freely to the outside and whose wall was perforated by a small hole (H) near the base of the rod. When this type of apparatus was used the nerve to be tested was laid along the rod in the usual manner but the end nearer the stopper, instead of being left within the chamber, was carried through the small hole into the hollow of the stopper as indicated by the dotted line in Fig. 2. Within the stopper it was placed on the platinum electrodes (E) of an induction apparatus. By this means the nerve could be stimulated well outside the respiratory chamber.

To prevent the free leakage of outside air into the respiratory chamber through the hole in the stopper, this opening was plugged with a mixture of kaolin and vaseline. This mixture made a snug fit between the nerve and the glass rim of the hole. It had been previously tested on nerve-muscle preparations and had been found not to interfere with nerve conduction even after twelve hours of application. Of course the open type of respiratory chamber possessed a higher rate of leakage than the closed one did. This rate varied with different experiments. When this type of chamber with the hole completely plugged was run without nerve the change from pH 7.78 to pH 7.36 required from two to three hours. A leakage correction was therefore introduced into all measurements made with the open type of chamber.

This general arrangement is believed to meet the criticism of Bayliss (1915, p. 379) as applied to Tashiro's apparatus in which the electrodes were within the respiratory chamber. In the apparatus just described the electrodes were so far outside the chamber that any heating and consequent liberation of CO_2 could have no effect on the gases in the respiratory chamber because of remoteness. Riggs (1919, p. 401) showed that when a nerve is stimulated not by elec-

tricity but by salts, an increase of CO_2 occurs. This increase, as he pointed out, could in no way be attributed to heat and hence its presence shows that the increase in Tashiro's experiments was not all dependent upon heat. But how much CO_2 may have been discharged in consequence of heat and how much may have been produced by increased metabolism cannot be stated. Hence the desirability of removing in some radical way this objectionable feature. It is believed that the apparatus used in these experiments accomplishes this end.

The calibration of the respiratory outfit employed in these experiments was first attempted by a method that I had previously devised for the Osterhout apparatus (Parker, 1922 a), but in consequence of the small capacity of the respiratory chamber used in these experiments I was unable to introduce into it with the necessary precision small measured quantities of CO_2. This effort, therefore, entirely failed.

Through the kindness of Dr. E. J. Cohn my attention was called to a method of calibration which had been devised by him to meet a similar condition in a research that was being carried on by Dr. G. B. Ray. This method, which has recently been described (Ray, 1924), met the requirements of my apparatus and I therefore adopted it. My thanks are due to Dr. Cohn and to Dr. Ray for their help in this important step.

The application of this method turns in part upon the fact that there is an even distribution of CO_2 throughout the apparatus at any given time irrespective of the proportion of fluid and of air contained in it. If, now, the indicator is dissolved in a weak aqueous solution of sodium hydrogen carbonate of known concentration, the amount of CO_2 per unit volume can be calculated for any pH value. In these experiments the concentration of sodium hydrogen carbonate used was 0.0001 mol. Under such conditions the amount of CO_2 at pH 7.36 was 0.0106 mg. per 10 cc. and at pH 7.78, 0.0040 mg. per 10 cc. Consequently the weight of CO_2 needed per 10 cc. to change from pH 7.78 to pH 7.36 is the difference between these two amounts or 0.0066 mg. (Ray, 1924, p. 510). The capacity of the closed respiratory chamber used in these experiments was found on measurement with mercury to be 30.4 cc. Hence the weight of CO_2 necessary to shift this chamber

from pH 7.78 to pH 7.36 was 0.0066 mg. × 3.04 or 0.020064 mg. The capacity of the open chamber used for the stimulation tests in these experiments measured in the same way was 30.1 cc. and the weight of CO_2 needed to make the appropriate shift in this case was by a similar procedure determined to be 0.019866 mg.

Ignoring the very small but continuous leakage of CO_2 into the apparatus from the outside, it may be said that when a piece of nerve causes the indicator in the closed apparatus to shift from pH 7.78 to pH 7.36 it must do so by discharging 0.020064 mg. of CO_2. As the time for this process is recorded in the experiment, all that is necessary for for the calculation of the rate of production of CO_2 is given. The weight of the nerve is also easily measurable and thus the rate of production of CO_2 can be readily expressed in relation to the amount of nerve concerned. In the following tabulations all the results pertaining to the production of CO_2 by nerve are expressed in milli rams of this gas per gram of nerve per minute.

Before turning to the actual results of the tests, it may be well to make a brief statement as to the sensitiveness of the apparatus. When a nerve tested in the closed chamber is found to discharge the amount of CO_2 requisite for the shift from pH 7.78 to pH 7.36 in 20 minutes, it can be shown that a difference in the tint of the indicator is just observable with certainty in about one minute after coincidence with the standard tube. As under these circumstances there are twenty such intervals in passing from one of the two pH values to the other, it is assumed that the method is sensitive to one-twentieth of the amount of CO_2 necessary for the total change or approximately 0.001 mg. This appears to be the limit of the amount of CO_2 that can be quantitatively detected by a respiratory chamber of the size used in these tests. The use of a smaller chamber would of course reduce in proportion to its size the amount of CO_2 that could be detected. In this way the apparatus is open to increased refinement. If Tashiro's statement (1913 b, p. 140) that the biometer used by him was sensitive to 1.0×10^{-7} grams of CO_2 (= 0.0001 mg.) is correct, his method must have been about ten times as sensitive as the one I used.

III. OBSERVATIONS.

When a freshly prepared lateral-line nerve from the dogfish is tested for CO_2 in the closed type of respiratory apparatus, this gas can be shown to be discharged from the nerve at first in considerable quantities and afterwards in much smaller amounts. The discharge is at first in the nature of a gush after which there follows a steady outflow for a number of hours with only slight diminution. This is well illustrated in nerve I (Fig. 4). As expressed in milligrams of CO_2 per

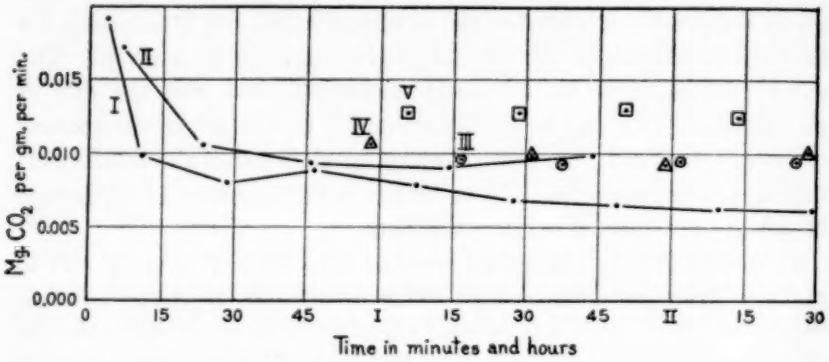

FIG. 4. Plottings of the respiratory rates in milligrams of CO_2 per gram of nerve per minute of five lateral-line nerves from the dogfish. The readings from Nerves I and II are represented by dots connected by lines; those from Nerve III are in circles, from Nerve IV are in triangles, and from Nerve V are in squares. Temperature about 23°C.

gram of nerve per minute this nerve showed an initial rate of 0.0192 which was followed by a decline first to 0.0098, and then to 0.0080, after which followed a slight increase to 0.0089, and then a gradual decrease to 0.0063. Essentially the same course is shown in Nerve II. This type of discharge was characteristic of all fresh nerves in these tests; an initial gush that lasted through about half an hour or more followed by a steady outflow with very gradual decline that extended in some instances over a period of as much as 8 hours. This decline was ordinarily succeeded by disturbances that were probably due to bacterial invasion; they were quite irregular and obscured the remaining steps of the process. The type of discharge just described has been noted by several previous observers (Tashiro, 1913 a, p.

117; Moore, 1918, p. 35; and Riggs, 1919, p. 401). Its significance is not wholly clear for the source of the CO_2 concerned has not been definitely determined.

There is every reason to believe that a nerve freshly removed from an animal such as a dogfish is taken from surroundings richly impregnated with CO_2 (Bayliss, 1915, p. 379). Such a nerve, therefore, would probably be well saturated with this gas and on exposing the nerve to the air the CO_2 would naturally escape from it at first rapidly and then more slowly till an equilibrium would be reached. In this way the relatively sudden gush of CO_2 followed by the more gradual discharge, as already described, might be explained. But a closer

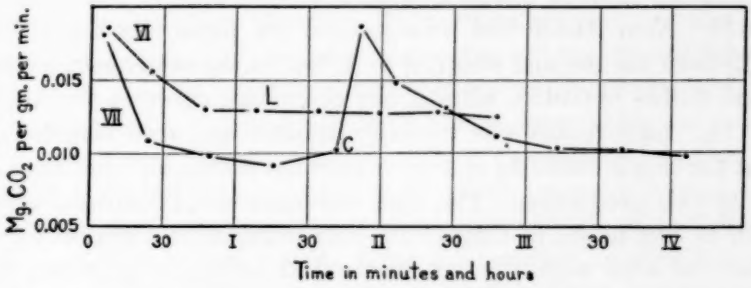

FIG. 5. Plottings of the respiratory rates in milligrams of CO_2 per gram of nerve per minute of two lateral-line nerves from the dogfish. Nerve VI, after its fourth test, was lifted (L) from the rod, returned to it, and then tested further. Nerve VII, after its fifth test, was cut (C) and then tested further. Temperature about 23°C.

inspection of the rates of escape of CO_2, as illustrated, for instance, by nerve I (Fig. 4), shows that though the gush might be considered the result of the exposure of a highly saturated nerve to air, the steady outflow cannot be thus explained. This outflow maintains itself at too high a level and for much too long a time to be interpreted as the final steps in the escape of CO_2 from such a reservoir as a nerve might be. The course of this outflow implies at once a continuous slow production of CO_2 within the nerve itself and thus supports the belief that the CO_2 at this stage of the discharge is not merely gas escaping mechanically from temporary storage, but a true excretion from the nerve.

It is also possible that the gush itself may be of this nature, for it immediately succeeds the preparation and handling of the nerve, operations that may be highly stimulating and hence productive of CO_2. To test the effects of preparation on a nerve several lines of experimentation were followed. Nerves were prepared in the usual way, tested, and, after they had arrived at the period of steady outflow, they were handled in that they were lifted from the rod in the respiratory chamber, returned to it, and then retested. In no case as a result of this treatment was the amount of discharged CO_2 increased to a measurable degree. This is well illustrated in nerve VI (Fig. 5). The rate of this nerve was at the beginning 0.0180 mg.; it then fell through 0.0156 to 0.0131, 0.0130, 0.0129, and so on to 0.0125. After 0.0130 had been reached the nerve was lifted (Fig. 5, L) from the rod and returned to it, but as the subsequent records show, 0.0129 to 0.0125, without any observable effect on the output of CO_2. Such results were regularly obtained and show conclusively that the simple handling of a nerve does not noticeably alter the rate of its CO_2 production. The high resistance of cold-blooded nerve, such as frog nerve, in comparison with mammalian nerve to the ill effects of even such vigorous mechanical action as stretching has already been pointed out by Forbes and Ray (1923, p. 461).

In a second set of tests the dogfish nerve, after it had arrived at the stage of the steady outflow, was cut in many places instead of being merely handled. This treatment yielded a very striking increase of CO_2 as shown by nerve VII (Fig. 5). This nerve began with an initial rate of 0.0176 mg. which soon fell to approximately 0.0100. After the fifth test on this nerve it was cut (Fig. 5, C) in sixteen places with the result that at the next test its rate had risen to 0.0186, higher than its initial rate. Recovery from this state was as rapid as from the initial gush and to approximately the same level (0.0100). Cutting, therefore, unlike mere handling calls forth an excessive discharge of CO_2 which, however, lasts over only a short period, like the initial gush.

Does cutting a live nerve excite metabolism or merely increase the surface through which stored CO_2 may escape? The latter opinion seems to be supported by Moore's observation (1917, p. 18), which I can confirm, that when a nerve is cut and put under a purple solution

of phenolsulphonephthalein, the ends of the nerve become bright yellow showing that they are very acid, probably from CO_2. But this is not the only part of the nerve that gives out CO_2. If a long piece of freshly prepared nerve is put in a respiratory chamber whose stopper is provided with a hole and the nerve is placed in such a way that its two cut ends are outside the respiratory chamber so that only an uncut loop of the nerve lies on the rod within the chamber, the same initial gush of CO_2 occurs as it does when the whole nerve is within the chamber. In one example of this kind the sequence of rates was as follows: 0.0231, 0.0142, 0.0131, 0.0121, 0.0120. Hence it is clear that the initial gush is not entirely through the cut ends of the nerve but must be thought of as resulting from a lateral escape from the nerve as well as from its ends. If, now, such a nerve as that just described is vigorously pressed or almost crushed but without injuring it to the extent of exposing much new surface, a new gush of CO_2 can be evoked. This was seen in the nerve from which the records just given were taken. After this nerve had subsided to the rate 0.0120, it was vigorously manipulated, though not really crushed, with the result that its carbon dioxide output immediately arose to 0.0247 and then fell again to 0.0119. Thus it is evident, as already maintained by Moore (1918, p. 35; 1921, p. 24), that vigorous manipulation and crushing increase the CO_2 output of a nerve to a striking degree. Although it is probable that some of the CO_2 that escapes from a prepared nerve has not been produced in the nerve but may have come from adjacent tissue and been held in the nerve as in a reservoir, it is certain, as the preceding results show, that the amount of such CO_2 must be relatively very small and that the principal volume of this gas in the initial gush and probably all of it during the steady outflow are the products of local metabolism within the nerve.

In this connection the CO_2 output of a nerve killed in boiling water is not without significance. If a freshly prepared nerve is steeped in boiling water one minute and immediately thereafter tested, it will be found to give out CO_2 at first at a low rate, approximately 0.0025 mg., and then at diminishing rates for about two hours after which no more CO_2 can be detected. These observations agree with those of Tashiro (1913 a, p. 116) who was unable to discover any striking amount of CO_2 from nerve killed by boiling. Moore (1917, p. 18)

also noted that a nerve killed by chloroform or by heat did not increase the acidity of the indicator in which it was temporarily immersed. These results show clearly that relatively soon after the death of the nerve the production of CO_2 falls very low and soon ceases altogether. The living nerve of a dogfish, however, will continue to produce this gas for at least eight hours after its removal from the fish's body, and this steady outflow, as already indicated, may be regarded as a fair measure of the metabolism of a quiescent nerve.

Such being the case it would be natural to suppose that the amount of CO_2 given out by a nerve would be in proportion to its weight. This relation has from the beginning been implied in the present discussion and, as might be expected, holds true on experimental test. Thus two nerves, A and B, were measured together and apart. nerve A was 10.2 cm. long and weighed 118 mg.; nerve B was 9.4 cm. long and weighed 82 mg. The nerves were tested in the closed respiratory chamber. To produce the amount of carbon dioxide for the necessary shift in this chamber (0.020064 mg.) required 1430 seconds for nerve A, 1990 seconds for nerve B, and 817 seconds for the two nerves together. Multiplying the weights of the nerves by the time required by each the following numbers are obtained: for nerve A 168,740, for nerve B 160,180 and for both 165,400 numbers that are in approximate agreement and that show that the amount of CO_2 discharged by a nerve is in proportion to its weight.

To obtain some quantitative statement of the rate of CO_2 production in quiescent dogfish nerve, records have been taken from nerves during the first two hours after the disappearance of gush. These records were obtained at a temperature of approximately 23°C. and are included in Fig. 4; they come from five nerves. Two of these nerves are numbers I and II already referred to and are represented by graphs in Fig. 4. The three remaining nerves, III, IV, and V, are also shown in Fig. 4 but merely by points, those of nerve III within circles, of nerve IV within triangles, and of nerve V within squares. I have assumed that all the records that lie between the 45 minute line and the 2 hour and 30 minute line in Fig. 4 may be reasonably regarded as representing the metabolism of quiescent nerve. Within this limit those from nerve I range from 0.0089 mg. to 0.0063 mg. and average 0.0071 mg.; from nerve II they average 0.0094 mg.; from

nerve III 0.0095 mg.; from nerve IV 0.0100 mg.; and from nerve V, the highest of all, 0.0128 mg. The general average for all five nerves is 0.0095 mg. Hence it may be stated that the quiescent lateral-line nerve of the dogfish discharges CO_2 at the average rate of 0.0095 mg. per gram of nerve per minute.

This determination stands somewhat higher than those reported by Tashiro (1913 a, pp. 112, 127). According to this investigator the nerve of the spider crab produces 6.7×10^{-7} gms. of CO_2 per 10 mgs. of nerve per 10 minutes or, expressed in the terms used in this paper, 0.0067 mg. of CO_2 per gram of nerve per minute. Frog nerve, according to Tashiro, produces 5.5×10^{-7} gms. of CO_2 per 10 gms. of nerve per 10 minutes, or 0.0055 mg. of CO_2 per gram of nerve per minute. Although both these determinations lie somewhat below mine, all three are obviously so close that they may be regarded as of the same order of magnitude and undoubtedly represent a close approximation to the true metabolic rate of quiescent cold-blooded nerve.

Is the rate of CO_2 production in the quiescent lateral-line nerve of the dogfish changed by stimulation? To answer this question the following tests were carried out. Long stretches of nerve were set up in the open type of respiratory chamber and, after the period of gush had passed, they were alternately stimulated by an induction apparatus and allowed to remain quiescent. As already stated the electrodes used for such operations were situated outside the respiratory chamber in the hollow stopper of that apparatus (Fig. 2). In all, four nerves were tested and in each instance a reading was first taken for the resting state after which electric stimulation was applied and a reading was taken while this was in progress. The nerve was then allowed to remain quiescent for from ten to fifteen minutes after which another reading, assumed to represent the quiescent state, was taken and again on stimulation a last reading. Thus four readings were obtained from each of the four nerves. These readings and the resulting averages are given in Table I.

As is shown in Table I all four nerves gave evidence of increased rates of CO_2 production during stimulation as compared with quiescence. The average rate of CO_2 production during quiescence was 0.01043 mg. CO_2 per gram of nerve per minute, and during stimulation

0.01208 mg., an increase in rate of 0.00165 mg. In percents the increase varied on the average from 14.3 to 16.7 of the rate in quiescence and gave a general average of 15.8 per cent. The increase though slight in amount, was characteristic of all the nerves tested. It cannot, I believe, be attributed to the heating of the nerve by the stimulating current, as suggested by Bayliss in his criticism of Tashiro's results, for, as already stated, the part of the nerve to which the current was applied was well outside the respiratory chamber.

As a check on the tests just recorded I steeped a nerve in boiling water for one minute and then subjected it to the kind of test just described for living nerve. During about two hours this nerve continued to give out a very small amount of CO_2 which beginning at 0.0020

TABLE I.

Rates of CO_2 Production in Milligrams of CO_2 per Gram of Nerve per Minute of Four Lateral-Line Nerves of the Dogfish Alternately Unstimulated and Stimulated.

No. of nerve.	State of nerve.				Averages.		Diff.	Diff. %.
	Unst.	Stim.	Unst.	Stim.	Unst.	Stim.		
1	.0116	.0138	.0114	.0125	.01150	.01315	.00165	14.3
2	.0096	.0115	.0092	.0103	.00940	.01090	.00150	16.0
3	.0118	.0130	.0112	.0138	.01150	.01340	.00190	16.5
4	.0090	.0113	.0096	.0104	.00930	.01085	.00155	16.7
General averages.....					.01043	.01208	.00165	15.8

mg. per gram of nerve per minute dropped eventually to nothing. This amount was never increased on stimulation but showed a steady decline. The contrast between the responses on stimulation of a living nerve and of a nerve killed by boiling is well shown in Fig. 6 in which are plotted the records of nerve 4 in Table I and those of the nerve killed in boiling water. The solid lines represent periods of quiescence, the dotted lines those of stimulation. As already stated, with living nerve each period of stimulation was accompanied with an increase in respiratory rate, but with the boiled nerve the periods of stimulation showed no such increase but the CO_2 steadily fell off till none could be detected. From these observations I conclude that the increase in the rate of production of CO_2 on stimulating a living

nerve is due to increased metabolism and is not occasioned, as Bayliss suggested, by experimental error.

The increased rate of CO_2 production on stimulation as detailed in the present account is not in agreement with the results of Tashiro or of Moore. Moore (1919 a, p. 66; 1919 b, p. 617; 1921, p. 24), using the method of immersion on frog nerve, was unable after 30 minutes of stimulation to observe any increase of rate in the output of CO_2. But Moore states that his method was capable of detecting only about a 10 percent difference. The increase that I have found, 15.8 percent, is so near this that perhaps even this increase failed to show itself in Moore's procedure. It is also possible, though I think

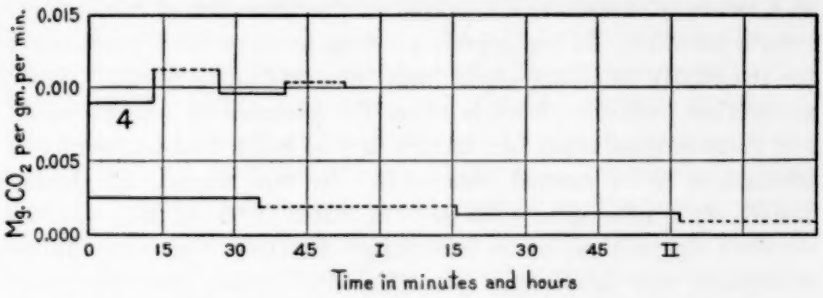

FIG. 6. Plottings of the respiratory rates in milligrams of CO_2 per gram of nerve per minute of two lateral-line nerves from the dogfish. The upper plotting (4) is from Nerve 4 in Table I and the lower one from a nerve killed by immersion in boiling water for one minute. Both nerves were alternately quiescent, represented by the full line, and stimulated, represented by the dotted line. Temperature about 23°C.

it not likely, that the rate of CO_2 production on stimulation in the frog nerve, which was used by Moore, is lower than that in the dogfish nerve, which I used. This question, however, calls for further investigation.

With Tashiro's conclusions the case is quite different. According to this investigator the increase of rate in CO_2 on stimulation is very high, with the nerve of the spider crab 100 to 200 percent and with that of the frog 200 to 300 percent. These increases are relatively so enormous that I feel sure that unless nerves are prodigiously diverse, I should have easily discovered by the method I used differences of this order of magnitude. But of such increases I have seen in the dogfish nerve not the least evidence. How the disparities between

Tashiro's results and mine are to be reconciled is not easy to state. So far as I can judge the method I have used is thoroughly sound. It enabled me to distinguish at least 0.001 mg. of CO_2, and the amount involved in determining an increase of rate of 15.8 percent is a little over 0.0025 mg. or about two and a half times this minimum. Had there been an increase of even 100 percent, not to mention 200 or 300 percent, I am sure I should have observed it, but as this investigation shows no increase of this proportion was ever met with. I do not believe that the nerves tested by Tashiro differ in so fundamental a way from the one studied by me. I suspect that the difficulty lies in the quantitative side of the biometer. The success of this apparatus as a means of measuring CO_2 turns on the formation of barium carbonate particles. In my experience these particles are formed much too irregularly to give a sound basis for quantitative results. Their production probably depends upon the presence of foreign nuclei and these or some other like factors vary so much that uniformity of production never seemed obtainable. In this respect, as already stated, my results agree with those of Adam (1921, p. 362) who has declared the method to be remarkably sensitive from a qualitative standpoint but insufficient quantitatively. I am therefore forced to set down the excessive increases of CO_2 attributed by Tashiro to nerve stimulation as due to an insufficient method. It is nevertheless remarkable that his determination of the CO_2 output for quiescent nerve should fall so close to mine.

As a result of this study on the lateral-line nerve of the dogfish I concluded that this nerve produces when quiescent approximately 0.0095 mg. of CO_2 per gram of nerve per minute and that this amount is increased by about 15.8 percent on stimulation.

What tissue in the nerve is the source of this CO_2 remains to be ascertained. Bayliss (1915, p. 379) pointed out the obvious fact that a nerve contains, beside nerve fibers, connective tissue and the like and that it is evident that this non-nervous tissue must respire. Consequently some, it not all, of the CO_2 given out by a quiescent nerve must come from this source. What proportion in a quietly respiring nerve is derived from its non-nervous elements and what from its nervous components cannot be stated. It is a common belief that the nucleated part of a cell is necessary for its respiration (Meyer-

hof, 1924, p. 11). Since the strictly nervous portions of a nerve trunk are the axis cylinders and since these represent in preparations such as were used in this research, parts of nerve cells separated from their nuclei, there has grown up an opinion that such axis cylinders cannot respire. Those who hold such a view would probably regard all the CO_2 that emanates from a quiescent nerve as due to the activity of its non-nervous components, connective-tissue cells, etc. I frankly admit that I have no evidence to show that such is not the case. But however this may be for quiescent nerve, it must be admitted, I believe, that the increase of CO_2 due to the stimulation of a nerve is from a strictly nervous source, for it is not remotely probable that stimulation as carried out in this research could influence connective tissue, blood vessels, or the like, in such a way as to produce at a distance of a few centimeters the increased CO_2 output recorded in this investigation. Hence I conclude that the 15.8 percent increase of CO_2 in the stimulated nerve of the dogfish is from strictly nervous sources and not from non-nervous elements. This amount, in my opinion, represents CO_2 that is the product of functionally active axis cylinders and their appended parts, such as the medullary sheaths, and is not due to the increased activity of the Schwann sheath-cells, the connective tissue elements, blood vessels, or other non-nervous elements. If this view is admitted, and I can see no reason why it should not be, it is also probably true that some at least of the CO_2 given out by a quiescent nerve comes from the same strictly nervous source as the excess excreted by the stimulated nerve does and represents the normal metabolism of this tissue in its quiescent state.

What proportion the excess CO_2 from stimulated nerve bears to the total CO_2 of strictly nervous origin cannot of course be stated. Tashiro has apparently assumed that all the CO_2 from a nerve was from nervous sources and from this standpoint he has estimated an increase of 200 to 300 percent on stimulation. This estimate led Mathews (1915, p. 590) to declare that nerve is perhaps the most actively metabolic tissue in the body. If, however, the rate of CO_2 increase is relatively low, as my determinations seem to indicate, nerve may not prove to be so exceptional. In a rough way I have tested in my apparatus pieces of fresh muscle and of fresh brain from the dogfish and I have found that the rate of CO_2 production in quiescent muscle

is almost three times that of quiescent nerve and in pieces of brain it is over four times that of nerve. From the observations of Moore (1918, p. 39) frog muscle appears to give out CO_2 at a rate of from three to seven times that of frog nerve and frog medulla from five to ten times that of frog nerve. Although these determinations, as well as my own, are avowedly rough and yield no certain criteria as to the respiration of pure tissue, they are in fair agreement and indicate that nerve has not an exceptionally high rate of respiration but on the contrary exhibits perhaps a lower one than that of many other tissues.

IV. GENERAL DISCUSSION.

Although it is not my intention to discuss in any detail the nature of the nervous impulse, it is nevertheless true that the observations set forth in this paper point to certain conclusions concerning this process and call for some comments particularly in its relation to oxygen, fatigue, and heat.

If active nerve excretes CO_2, it should be expected to absorb oxygen. Over two decades ago von Baeyer (1902) observed that frog nerve in an atmosphere of hydrogen or of nitrogen became inexcitable in from 3 to 5 hours and recovered excitability in air after from 3 to 10 minutes. This condition was shown to be dependent upon oxygen and has commonly been regarded as a kind of asphyxiation. Since its discovery the asphyxiation of nerve has been confirmed by many workers and has afforded the starting point within recent years of a number of important investigations (Tashiro and Adams, 1914; MacArthur and Jones, 1917; Gottschalk, 1920; Adam, 1921; Sheaff, 1922; Thörner, 1922 a, 1922 b; Cooper, 1923).

Assuming oxygen to be an essential element in nerve activity, the minimum amount of this gas needed in the case of any given nerve may be easily calculated on the basis of the CO_2 discharged by that nerve. Thus to produce 0.0095 mg. CO_2, the amount of this gas discharged by a gram of resting dogfish nerve in a minute, would require an oxygen supply of 0.0070 mg. The oxygen consumption of frog nerve, which is probably very close to that of dogfish nerve, has already been determined by Adam (1921) and by Sheaff (1922). Sheaff working with an apparatus, which was essentially a biometer modified

to meet the needs of oxygen determination, found that 10 mg. of quiescent frog nerve absorbed in 10 minutes from 0.434 to 0.76 $\times 10^{-5}$ grams of oxygen, or from 0.0434 to 0.076 mg. oxygen per gram of nerve per minute. This implies a rate of oxygen consumption approximately five to ten times that necessitated by the corresponding CO_2 discharge reported in this paper. Why Sheaff's figures should be so high is not wholly clear. In his description of his method he (1922, p. 47) states that in preparing and placing the nerve he worked *speedily*. Under such circumstances the nerve must have been in the period of CO_2 gush. If CO_2 and oxygen are related as might be expected, this period would also be one of excessive oxygen absorption which may possibly explain in part at least Sheaff's relatively high determinations.

According to Adam (1921) resting frog nerve absorbs only from 0.05 to 0.08 c.c. of oxygen per gram of nerve per hour, which when transposed to the form of expression used in this paper amounts to 0.012 mg. to 0.019 mg. of oxygen per gram of nerve per minute. These determinations are so close to what has been reported in this paper (0.007 mg.), that they afford a reasonable confirmation of the belief that the oxygen absorption and the CO_2 production in nerve are steps in one and the same general process (Lucas, 1917, p. 26).

With this conclusion in mind it is not surprising to find that both Sheaff and Adam record an increase of oxygen absorption on the stimulation of nerve. In Sheaff's studies the determinations are high compared with what would be anticipated from the CO_2 increase recorded in this paper. Adam's determinations, on the other hand, fall very close to the results herein reported. According to him a stimulated frog nerve absorbs an excess of oxygen amounting to 0.02 c.c. per gram of nerve per 15 minutes which is equivalent to 0.0019 mg. oxygen per gram of nerve per minute. This is so near to 0.0011 mg. oxygen, the weight calculated from the excess CO_2 determination given in the present paper as to amount almost to agreement and I therefore conclude that both in quiescent nerve and in stimulated nerve the weight of oxygen absorbed is directly related to the weight of CO_2 excreted.

If nerve exhibits a metabolism in which oxygen is absorbed and CO_2 discharged, why does it not show signs of exhaustion, as, for

instance, muscle does? The ability of nerve to respond continuously to stimulation has long been known and this fact has been urged repeatedly by those who favor a physical conception of transmission. It is, however, quite probable that nerve does tire but that the period of its exhaustion is extremely brief in that its recovery is very rapid. The refractory period of a nerve immediately follows the passage of an impulse and the striking peculiarity of this period is the well known inability of the nerve to respond to a renewed stimulus. During this period the nerve changes from the condition in which it cannot respond to stimulation to one in which it can; in other words, the refractory period is a period of recovery. It begins with all the signs of full exhaustion and passes on quickly to a condition of full recovery. This momentary exhaustion is in my opinion the sign of real nerve exhaustion. It is not a cumulative exhaustion like that of muscle but a momentary one. From this standpoint the activity of nerve with its brief period of tiring and its rapid recovery has been compared to the action of insect muscles in flight (Tashiro, 1915, p. 111) or better to the heart (Lasareff, 1924), in which what appears to be a process of continuous activity is made up of steps of momentary exhaustion and of quick recovery. Viewed from this standpoint active nerve as a consequence of its activity does tire, but its recovery is so quick that the tiring is shown only over a very brief period. This view of nerve exhaustion has already been expressed by Bayliss (1915, p. 390).

Whether the CO_2 liberated from stimulated nerve is produced in the passage of the impulse or during the immediately following process of recovery has not been ascertained. Judged from the standpoint of muscle physiology, the recovery process in nerve may be the one concerned with the production of CO_2 rather than the process represented by the impulse itself (Cooper, 1923), but on this problem I have no critical observation to offer.

If active nerve absorbs oxygen, discharges CO_2, and exhibits momentary exhaustion, its operations would appear to depend in part at least upon oxidation and might therefore be expected to be accompanied with an evolution of heat. As is well known Hill (1911) on tetanizing a nerve for 25 seconds failed to get evidence of an increase of temperature in the nerve by an apparatus that was sensitive to

about six millionths of a degree centigrade. How is the amount of heat that may be assumed in the production of 0.00165 mg. of excess CO_2 per gram of nerve per minute related to this very low limit set by Hill? If we assume the heat in the nerve to have been generated by the oxidation of some such substance as glucose, it can be shown by calculation that the production of 0.00165 mg. CO_2 may be expected to liberate approximately 0.0042 gram-calories of heat. This implies that any piece of nerve on being stimulated would produce in a period of one minute enough heat to raise its temperature about 0.0042°C. provided that the nerve was like water and that none of the heat produced in it escaped. Nerve doubtless requires about as much heat as water does to bring up its temperature a given amount and of course all the heat produced in nerve would eventually escape, but its dissipation would be relatively slow so that beginning with such a comparatively large amount as this reaction implies, a temperature difference capable of being measured ought to be within the possibility of observation. It is, however, conceivable that the chemistry of the nervous impulse may not involve the oxidation of such a substance as glucose, but may depend for its final step upon the oxidation of some almost completely oxidized compound which step would then liberate CO_2 with the least possible evolution of heat and thus greatly reduce this factor. It can be easily shown, for instance, that while the production of 0.00165 mg. CO_2 from glucose is accompanied by an evolution of 0.0042 gram-calories of heat, the same amount of CO_2 can be obtained by burning the much more completely oxidized oxalic acid with an evolution of only about 0.0011 gram-calories of heat or about one-fourth the former amount. In this way it is conceivable that the production of a given quantity of CO_2 may be accomplished under one set of circumstances with a much lower output of heat than under other circumstances. But although the nerve impulse may depend upon a much lower heat-producing process than the oxidation of glucose, it is highly improbable that an oxidation could be found that would reduce this operation to a level consistent with Hill's determination. Harvey (1919, p. 142), however, seems to imply such reduced processes in his discussion of heat production in bioluminescence. Here not enough heat was evolved to make a change of 0.0005°C. in the solution used. Harvey concludes,

not that heat is absent, but that the oxidation of the light producing substance, luciferin, is accomplished by a process that involves only a minimum of heat. But whether this is the true explanation of such conditions or not, there must be a limit to this kind of reduction and this limit in the case of nerve seems to have been exceeded by what Hill has shown to be the requirements of his own determination. In a recent paper by this author (Hill, 1921), however, in the course of a very illuminating discussion of the temperature coefficient of the nerve impulse he concludes that in nerve transmission "a chemical change interposes somewhere in the process." The bearing of this conclusion on the possibility of heat production in nerve action is not discussed probably because the whole subject is unsettled and ripe for reinvestigation rather than for speculation. In such reinvestigation it may be shown that nerve, like muscle, can store carbonates from which with a minimum of heat production CO_2 may be liberated by an acid formed in the nerve.

The comparison of a nerve impulse to the burning of a chain of gun-powder, though not very close, is nevertheless in many ways suggestive. In both oxygen is used, CO_2 discharged, and a progressive activity exhibited. But in nerve the oxidation is probably a step in restoration rather than part of the impulse proper, for the fresh nerve seems rather like a suspended weight which on being loosened by some agent corresponding to the stimulus falls, whereupon work must be done to return it to its initial elevation. It is this operation of return that involves in the nerve oxidation and the output of CO_2. Further, the nerve impulse is a change that progresses over the substance of nerve not by the spread of heat but probably by a change in electrical potential, as indicated by Lillie (1923), and in this respect also it differs from a chain of burning gun-powder. But however we may analyze such examples, it seems quite clear, as stated by Forbes (1923, p. 49), that the purely physical conception of the nerve impulse has ceased to have much weight and we must agree with Hill (1921, p. 334) that chemical change interposes somewhere in it.

V. SUMMARY.

1. A modified Osterhout respiratory apparatus for the detection of CO_2 from nerve is described.

2. The lateral-line nerve from the dogfish discharges CO_2 at first with a gush for half an hour or so and then steadily at a lower rate for several hours.

3. Simple handling of the nerve does not increase the output of CO_2; cutting it revives gush.

4. The CO_2 produced by nerve is not escaping simply from a reservoir but is a true nervous metabolite.

5. The rate of discharge of CO_2 from a quiescent nerve varied from 0.0071 to 0.0128 mg. per gram of nerve per minute and averaged 0.0095 mg.

6. Stimulated nerve showed an increased rate of CO_2 production of 15.8 percent over that of quiescent nerve.

7. The results of these studies indicate that chemical change is a factor in nerve transmission.

VI. LITERATURE.

Adam, N. K. 1921. Note on the Oxygen Consumption of Amphibian Muscle and Nerve. Biochem. J., **15,** 358.

von Baeyer, H. 1902. Das Sauerstoffbedürfniss des Nerven. Zeit. allg. Physiol., **2,** 169.

Bayliss, W. M. 1915. Principles of General Physiology. London.

Bodine, J. H. 1921 a. Factors Influencing the Water Content and the Rate of Metabolism of Certain Orthoptera. J. Exp. Zoöl., **32,** 137.

1921 b. Some Factors Influencing the Catalase Content of Organism. J. Exp. Zoöl., **34,** 143.

1922. The Effect of Light and Decapitation on the Rate of CO_2 Output of Certain Orthoptera. J. Exp. Zoöl., **35,** 47.

Cooper, S. 1923. The Rate of Recovery of Nerve in Asphyxia. J. Physiol., **58,** 41.

Forbes, A. 1923. The Physico-Chemical Basis of Psychic Phenomena. Science, **58,** 49.

Forbes, A., and L. H. Ray. 1923. The Conditions of Survival of Mammalian Nerve Trunks. Amer. J. Physiol., **64,** 435.

Gottschalk, A. 1920. Über den Mechanismus der Erstickung und Erholung des markhaltigen Kaltblüternerven. Zeit. allg. Physiol., **18,** 341.

Harvey, E. N. 1919. Studies on Bioluminescence. XI. Heat Production During Luminescence of Cypridina Luciferin. J. Gen. Physiol., **2,** 137.

Hill, A. V. 1911. The Absence of Temperature Changes during the Transmission of a Nervous Impulse. J. Physiol., **43,** 433.

1921. The Temperature Coefficient of the Velocity of a Nervous Impulse. J. Physiol., **54,** 332.

Hughes, J. S., and H. H. King. 1923. Physico-Chemical Basis of Psychic Phenomena. Science, **57**, 590.

Irwin, M. 1920. An Apparatus for Measuring the Production of Minute Quantities of Carbon Dioxide by Organisms. J. Gen. Physiol., **3**, 203.

Jacobs, M. H. 1920. A Simple Method of Measuring the Rate of Respiration of Small Organisms. Amer. Nat., **54**, 91.

Lasareff, P. 1924. Sur la cause physico-chimique de l'absence de la fatigue dans les centres nerveux au cours de leur action. Compt. rend. Acad. Sci., Paris, **178**, 1100.

Lillie, R. S. 1923. Protoplasmic Action and Nervous Action. Chicago.

Lucas, K. 1917. The Conduction of the Nervous Impulse. London.

Lund, E. J. 1919. A Simple Method for Measuring Carbon Dioxide Produced by Small Organisms. Biol. Bull., **36**, 105.

MacArthur, C. G., and O. C. Jones. 1917. Some Factors Influencing the Respiration of Ground Nervous Tissue. J. Biol. Chem., **32**, 259.

Mathews, A. P. 1915. Physiological Chemistry. New York.

Meyerhof, O. 1924. Chemical Dynamics of Life Phaenomena. Philadelphia.

Moore, A. R. 1917. Acid Development as the Result of Injury in Nervous Tissue. Proc. Soc. Exp. Biol. Med., **15**, 18.

1918. The Carbon Dioxide of Injury and of Respiration in Nervous Tissue. Proc. Soc. Exp. Biol. Med., **16**, 35.

1919 a. Electrical Stimulation and CO_2 Production in Nervous Tissue. Proc. Soc. Exp. Biol. Med., **16**, 66.

1919 b. The Respiratory Rate of the Sciatic Nerve of the Frog in Rest and Activity. J. Gen. Physiol., **1**, 613.

1921. Respiratory Rate in Nervous Tissue. Proc. Pathol. Soc., Philadelphia, **41**, 24.

Osterhout, W. J. V. 1918. A Method of Studying Respiration. J. Gen. Physiol., **1**, 17.

Osterhout, W. J. V., and A. R. C. Haas. 1918. A Simple Method of Measuring Photosynthesis. Science, **47**, 420.

Parker, G. H. 1922 a. The Calibration of the Osterhout Respiratory Apparatus for Absolute Quantities of Carbon Dioxide. J. Gen. Physiol., **4**, 689.

1922 b. The Excretion of Carbon Dioxide by Relaxed and Contracted Sea Anemones. J. Gen. Physiol., **5**, 45.

Ray, G. B. 1924. The Oxidation of Sodium Lactate by Hydrogen Peroxide. J. Gen. Physiol., **6**, 509.

Riggs, L. K. 1919. Action of Salts upon the Metabolism of Nerves. J. Biol. Chem., **39**, 385.

Sheaff, H. M. 1922. A Method for the Quantitative Estimation of Minute Amounts of Gaseous Oxygen and its Application to Respiratory Air. J. Biol. Chem., **52**, 35.

Tashiro, S., 1913 a. Carbon dioxide production from Nerve Fibres when Resting and when Stimulated. Amer. J. Physiol., **32**, 107.

1913 b. A New Method and Apparatus for the Estimation of Exceedingly Minute Quantities of Carbon dioxide. Amer. J. Physiol., **32,** 137.

1914. Carbon Dioxide Apparatus. III. J. Biol. Chem., **16,** 485.

1915. On the Nature of the Nerve Impulse. Proc. Nat. Acad. Sci., **1,** 110.

1917. A Chemical Sign of Life. Chicago.

1921. Can an Indicator Method be used for Measurement of CO_2 Production from Isolated Nerve? J. Biol. Chem., **46,** XV.

1922. Studies on Alkaligenesis in Tissues. I. Ammonia Production in the Nerve Fiber during Excitation. Amer. J. Physiol., **60,** 519.

Tashiro, S., and H. S. Adams. 1914. Carbon dioxide Production from the Nerve Fiber in a Hydrogen Atmosphere. Amer. J. Physiol., **34,** 405.

Thörner, W. 1922 a. Elektrophysiologische Untersuchungen am alterierten Nerven: I. Mitteilung. Die überlegene Erregungswirkung der aufsteigenden konstanten und Induktions-Ströme infolge Zunahme der anodischen Öffnungserregbarkeit und kathodischen Depression während der Erstickung. Arch. ges. Physiol., **197,** 159.

1922 b. Elektrophysiologische Untersuchungen am alterierten Nerven. II. Mitteilung. Sauerstoffentziehung und physiologischer Elektrotonus. Arch. ges. Physiol., **197,** 187.

Waller, A. D. 1896. Croonian Lecture. Observations on Isolated Nerve. (Abstract.) Proc. Roy. Soc., London, **59,** 308.

1897 a. Croonian Lecture. Observations on Isolated Nerve (with particular reference to Carbon Dioxide). Philos. Trans. Roy. Soc., London, B, **188,** 1.

1897 b. Lectures on Physiology. First Series. On Animal Electricity. London.

1910. Physiology the Servant of Medicine. London.